DEVELOPMENT OF BANANA BUNCHY TOP VIRUS RESISTANCE IN BANANAS: RNAi APPROACH

by

Benard Ouma Mware

BSc. (Agriculture) 2005, MSc. (Crop Protection) 2009 (University of Nairobi, Kenya)

Centre for Tropical Crops and Biocommodities

Science and Engineering Faculty

A thesis submitted for the degree of Doctor of Philosophy

Queensland University of Technology

2016

Keywords

Agrobacterium-mediated transformation, Banana, Banana bunchy top virus, embryogenic cell suspension, genotype, GUS, hairpin, *Musa*, resistance, RNAi, siRNA, transgenic plants, transgene.

Abstract

Banana bunchy top virus (BBTV), the causal agent of banana bunchy top disease (BBTD), is a circular, single-stranded virus belonging to the genus *Babuvirus*. BBTD is the most important viral disease known to affect banana and causes dramatic yield losses worldwide. Genetic engineering is likely the best approach to improve accepted banana varieties and molecular strategies using RNAi for engineered resistance to BBTV have recently been developed.

This PhD study addressed whether a RNAi strategy could be used to confer resistance to BBTV in commercially grown dessert cultivars of banana. A suite of hairpin expression cassettes targeting the South Pacific (SP) strain of BBTV DNA-R (Master Rep), DNA-M (movement protein) and DNA-N (nuclear shuttle protein) gene sequences were assembled in binary vectors. Using *Agrobacterium*-mediated transformation, these cassettes were transferred into banana embryogenic cells and 67 independent transgenic lines were established *in vitro*. Transgenic plants were confirmed to contain the hairpin cassette by PCR, multiplied in tissue culture and acclimatised in soil for small-scale glasshouse virus challenge. Plants were infected with BBTV (SP strain) using viruliferous aphids and monitored for symptom development and the presence of viral nucleic acids over time. Of the hairpins tested, those targeting the BBTV (SP strain) DNA-M gene were the most effective for virus resistance. The majority of lines expressing these RNAi cassettes (approximately 85% of lines) remained free of BBTD symptoms 12 weeks post challenge compared to the wildtype controls. Interestingly, the degree of resistance in these lines strongly correlated with the number of hairpin copies integrated in the banana genome, with low single copy plants providing more robust virus resistance compared to high copy number plants. Small 21 nucleotide RNAs with homology to the DNA-M gene were detected in resistant lines by northern hybridisation. The presence of these hallmark small RNA species strongly suggested resistance was the result of activation of the RNAi pathway in banana. Hairpin constructs targeting either BBTV (SP strain) DNA-R or DNA-N genes provided no resistance or very limited tolerance to the virus.

To determine whether hairpins can cross-protect against geographically diverse isolates of BBTV (between 85.5-90% homology), a small number of banana plants transformed with RNAi cassettes targeting BBTV (Asian strain) DNA-R and DNA-M were challenged with BBTV (SP strain). All plants tested were susceptible to infection and displayed typical BBTD symptoms 3 weeks post-challenge. This finding may reflect an inability to cross protect between BBTV strains but is far from conclusive considering (i) the small number (5 independent lines) of plants tested, (ii) the high (between 2 to 6) number of integrated hairpin copies detected in these lines and (iii) the majority of these lines contained hairpins against the less effective DNA-R gene target.

Alternative strategies for mechanically infecting banana plants with BBTV were investigated as a simple alternative to using the natural aphid vector. Greater than component length nucleic acids were generated for the six BBTV DNAs by rolling circle amplification (RCA) and prepared for delivery as either naked DNA or cloned into a suitable vector. Two methods of delivery into banana leaves or pseudostems were trialled including biolistic delivery on gold micro-carriers and *Agrobacterium*-inoculation (agro-inoculation). Despite optimisation of the biolistic delivery parameters using a GUS reporter gene enzyme, no BBTV infections were established using micro-projectile delivery with the various BBTV DNA forms. Also, plants agro-inoculated with the six cloned BBTV components showed no evidence of BBTV infection while those agro-inoculated with a badnavirus control, *Banana streak Mysore virus*, displayed symptoms typical of badnavirus infection.

There is evidence to suggest that some banana cultivars have varying degrees of tolerance/resistance to BBTV and that this may be genotype dependent, with an emphasis on the B genome. To investigate this in greater detail a diverse collection of 11 banana genotypes were established in the glasshouse and challenged with BBTV. Symptoms were scored and viral nucleic acids detected by PCR, 12 weeks post-challenge. Of those tested, Gros Michel (AAA genotype), Saba (ABB genotype), Khae Phrae (AA genotype), Ney Poovan (AB genotype) and Butuhan (BB genotype)

showed high levels of resistance to the virus and remained free of symptoms and viral nucleic acids. These findings suggested the B genome is not absolutely essential for natural BBTV resistance in banana. It is also the first report of a diploid AA banana with natural immunity to the virus.

Quantitative PCR analysis of the six BBTV DNA components in infected plants established (i) each circular DNA accumulates to different levels, (ii) the DNA-N component is the most abundant of all six components, (iii) amounts of each DNA component remain relatively steady over a course of an infection and (iv) the relative levels of each component are conserved between different banana genotypes. When the relative DNA levels were adjusted to a baseline of 1 copy (using DNA-S which had the lowest value throughout) an average genome formula for BBTV was determined to be S^{1} C^{2} M^{6} R^{44} $U3^{59}$ N^{692}. The significance of this strong bias towards the replication and abundance of some components over others and whether this is a virus or host-driven phenomena, however, remains unclear.

This study has provided a useful molecular guide for the future genetic improvement of bananas with resistance to BBTV. It has proven that RNAi is an effective and robust resistance strategy but that the choice of target gene is paramount to its success; the DNA-M gene being a particularly attractive hairpin target. Further, this work has identified strong natural BBTV resistance in banana cultivars containing both A and B genotypes and shown the six essential DNA components of the virus accumulate to a formula that is consistent throughout infection and independent of banana genotype.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

°C	degrees Celsius
µL	microliter/s
µM	micromolar
AGO1	Argonaute protein 1
BAP	6-benzylaminopurine
BLAST	basic local alignment search tool
bp	base pair/s
BRM	bacterial re-suspension media
cDNA	complementary DNA
Clink	cell cycle link protein
cm	centimetre/s
CP	coat protein (capsid protein)
CR	common region
CR-M	common region major
CR-SL	stem loop common region
css	circular single strand
CTAB	cetyltrimethyl ammonium bromide
DEPC	diethylpyrocarbonate
dH_20	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate

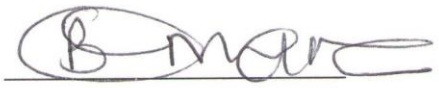
ds	double-stranded
dsDNA	double-stranded DNA
E. coli	*Escherichia coli*
ECS	embryogenic cell suspensions
EDTA	ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organisation
g	gram/s
g	relative centrifugal force in units of gravity
GUS	β-glucuronidase
h	hour/s
L	litre/s
LB	Luria-Bertani
M	Molar
min	minute/s
mM	millimolar
MP	movement protein
mRNA	messenger RNA
MW	molecular weight
NAA	α-naphthalene acetic acid
nm	nanometre/s
no.	number
Nos	gene encoding nopaline synthase
NSP	nuclear shuttle protein
nt	nucleotide/s
ORF	open reading frame

PCR	polymerase chain reaction
pH	- log (proton concentration)
pmol	picomole/s
RBR	retinoblastoma-related
RCA	rolling-circle amplification
RCR	rolling-circle replication
REn	replication enhancer
RNA	ribonucleic acid
RNAi	RNA interference
RNaseA	ribonuclease A
RSSs	RNA silencing suppressors
RT	reverse transcriptase
RT-PCR	reverse transcriptase PCR
s	second/s
SDS	sodium dodecyl sulphate
siRNA	small interfering RNA
SP	South Pacific
spp	species
ss	single-stranded
ssDNA	single-stranded DNA
ssp	subspecies
TAE	Tris acetate EDTA
TBSV	tomato bushy stunt virus
TE	Tris-EDTA
TrAP	transcriptional activation protein

Tris	tris (hydroxymethyl)aminomethane
Tween 20	polyoxyethylene (20) sorbitan monolaurate
U	unit
Ubi	ubiquitin
UTR	untranslated region
UV	ultraviolet
V	volt/s
v/v	volume per volume
VN	Vietnam
VSR	viral suppressors of RNA silencing
W/V	weight per volume
WT	wild type
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronide-cyclohexylamine salt

Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

Signature: 

Date: May 2016

Acknowledgements

I would like to thank my supervisors for the support during the PhD journey. Your relentless effort, insight and encouragement made such a big difference. In particular, I must acknowledge Distinguished Professor James Dale and Professor Rob Harding for giving me a chance to undertake my study within the banana research group. I remember Rob a number of times telling me that he had no book shelf for failed theses. It was really encouraging to hear this Rob. Thank you. I will never forget the everyday consultations and brain storming moments I had with Dr Anthony James. Thank you Anthony for the times you spared to come along with me during the screening work and the enormous support during my studies. Thank you Professor James Dale for funding the cost of the project and immense support you offered during the PhD work.

I also recognise the support of the entire CTCB team, in particular Dr Julia Bally who assisted with the Northern blot analyses and my colleagues (Jacqui, Betty, Mumbi, Bosibori and Steve Buah) with whom we walked together during my studies. Many thanks to the CTCB administrative team, especially Diana O'Rourke, for the help and support offered over the study period.

Gratitude to the Australian Government for the scholarship offered through the Australia Awards scholarship program within the Department of Foreign Affairs. I also thank Professor James Dale, CTCB and QUT for the scholarship towards the end of the research work.

Great appreciation goes to my family (Emily, Furaha and Jayden), Dr. Amolo and his family for sacrificial input into my education, Mum and Dad for the patience and support during my studies. Thank you Emily for the encouragement and support you offered.

Glory is to God in the highest for his favour, insight and for the blessing of sound mind and protection.

CHAPTER 1

LITERATURE REVIEW

1.1 Banana and plantains

Banana and plantains (*Musaceae*, *Zingiberales*, *Musa* spp.) are believed to have originated in Southeast Asia and the western Pacific. They are among the first crops to be domesticated and are now widely distributed throughout the tropics and subtropics where they constitute a major staple food for millions of people (Davey *et al.*, 2009; De Langhe *et al.*, 2009). Globally they are the most important fruit coming fourth in the list of global food crops with approximately 80% of production being used for local consumption (Novák *et al.*, 2014). Although only 10% of banana production is traded internationally, a total of 16.5 million tonnes (FAO, 2014) valued at US$16.5 billion was traded in the global export market (FAO, 2014). Bananas are a vital source of income to several countries (UNCTAD, 2012). It plays a pivotal role as a basic food source throughout more than 120 countries with a consumption of up to 400 kg per person per year in some countries (FAO, 2010). In nutritional terms, banana fruit provides energy, minerals and vitamins (Harish *et al.*, 2008) making it second only to citrus by nutritional value among fruits (UNCTAD, 2012). In addition to providing fruit for human consumption, several other products can be derived from banana plants including fibre, vegetables (male bud), beer, wine and vinegar.

1.2 Banana genetic diversity

Musa spp. have been classified based on four genome types corresponding to genetic constitution of four wild Eumusa species namely A genome *Musa acuminata* (2n = 2x = 22), B genome *Musa balbisiana* (B-genome, 2n = 2x = 22), S genome *Musa schizocarpa* (2n = 2x = 22) and T genome which is the Australimusa species (2n = 2x = 20). DNA molecular markers have been used to describe and categorise subspecies within the *M. acuminata* into four subgroups namely banksii, zebrina, malaccensis and burmannica/burmannicoides (Boonruangrod *et al.*, 2009; Mahendhiran *et al.*, 2014; Raboin *et al.*, 2005). On the contrary, *M. balbisiana* has not had subspecies classified despite evidence of interspecific genetic variation (Wang *et al.*, 2007). Members of the Australimusa section vary from the acuminata and balbisiana species in their chromosome number (2n = 20), comprising seven species with one edible parthenocarpic type called ‘Fe’i bananas. It is the Fe’i group members that

are distinctively known for their upright fruit and sometimes high levels of provitamin A carotenoids (Davey *et al.*, 2009; Englberger *et al.*, 2006).

Cultivated bananas are parthenocarpic, seedless, vegetatively propagated hybrids believed to have arisen from hybridisations between wild diploid *M. acuminata* and *M. balbisiana* species (Kumar *et al.*, 2015; Ortiz, 2013). Consequently, intraspecific hybridisations within *M. acuminata*, and cross hybridisations between *M. acuminata* and *M. balbisiana* have produced diverse combinations of A- and B -genomes commonly categorised into six groups namely AA, AAA, AB, AAB, ABB and ABBB (Heslop-Harrison & Schwarzacher, 2007). Most of the edible cultivars are allopolyploid triploids further grouped depending on genome constitution; that is dessert (AAA), plantain (AAB) and cooking (ABB) bananas (Heslop-Harrison & Schwarzacher, 2007). Of interest is the fact that there is no clear cut division between parental A- and B- chromosomes during hybridisation and that there is evidence supporting pairing and recombination between homologous A- and B- chromosomes (D'Hont *et al.*, 2012; Jeridi *et al.*, 2011). This is not unique to *Musa* spp. as similar events are reported to occur in allopolyploids of other species (Gaeta & Pires, 2010). It is plausible that modern day interspecific triploids may have resulted from one or more steps of (re)combination and swapping of chromosomal segments between the A- and B-genomes (De Langhe *et al.*, 2010; Jeridi *et al.*, 2011; Perrier *et al.*, 2009). Thus, a majority if not all *Musa* spp cultivars may possess genomes consisting of different segments of the A- and B-genome. It is thought that this kind of hybridisation process among *M. acuminata* subspecies may have contributed to the evolution of the edible AA and AAA types. One of the effects of these recombination events is that the resulting hybrid genomes contain a proportionate number of A- and B-genome alleles (Henry *et al.*, 2011). This situation complicates genetic studies of trait inheritance and challenges the development and application of molecular marker technologies (De Langhe *et al.*, 2010).

1.3 Threats to banana production and improvement

Despite banana's rich genetic diversity, its existence is threatened by diseases, the ever-changing climate and farming practices (Christelová *et al.*, 2011). Today, more than 90% of dessert bananas for commercial export are generated from one cultivar called Cavendish (AAA). The reliance on a single cultivar limits genetic variation in production systems and

may result in disease pandemics. However, it is believed that wild banana, *M. balbisiana* Colla, contains important genetic resources that can be utilised in banana breeding programs due to several agriculturally advantageous abiotic and biotic characters, such as cold- and disease-resistances (Wang *et al.*, 2007). Despite this belief, all known commercial banana varieties are highly susceptible to fungal and bacterial pathogens as well as nematodes, viruses, and insect pests (Lu *et al.*, 2011). One such disease pandemic in the 1950s, resulted from Panama disease or 'Fusarium wilt', caused by the soil-borne fungus *Fusarium oxysporum* f.sp. *cubense* which caused major losses to production of 'Gros Michel' (AAA) plantations (Molina *et al.,* 2010). Consequently this cultivar was replaced by the Fusarium-resistant 'Cavendish' cultivars, but with the recent emergence of a new strain of *F. oxysporum* (known as 'Tropical race 4'), this natural resistance in Cavendish is also under threat (Davey *et al.*, 2013). Similarly, bunchy top disease inflicted devastating effects in the banana industry in Australia in the 20th century, but due to strict quarantine regulations and enforcement this disease has mostly been contained (Dale, 1987; Magee, 1927). To date, there is no report of bunchy top virus resistance in the entire *Musa* germplasm. As a result, there is need for sustainable solutions to curb banana disease threats by identification and introduction of novel resistance loci and generation of new disease resistant cultivars. However, the sterility and ploidy nature of commercial banana varieties make breeding a time consuming venture (Davey *et al.*, 2013).

1.4 Banana bunchy top disease

Banana bunchy top disease (bunchy top), caused by *Banana bunchy top virus* (BBTV), is the most serious viral constraint to banana production worldwide. The disease occurs in many countries of Africa, Asia and the South Pacific (Fig. 1.1) (Amin *et al.*, 2008; Kavino *et al.*, 2011; Kumar *et al.*, 2011; Selvarajan *et al.*, 2010). Bunchy top was first recognised in Fiji in 1889, followed by Taiwan (1890), Egypt (1901) and Australia in 1913 (Magee, 1927). Bunchy top epidemics have occurred in Fiji (1927), India and Egypt (1953), Taiwan (1961), Philippines (1979) and China (1979) and more recently in Pakistan (Amin *et al.*, 2008) and sub-Saharan Africa (Kumar *et al.*, 2011).

The symptoms of bunchy top include narrowing of the leaves, development of chlorotic margins, severe stunting and the occurrence of discontinuous dark-green streaks on the leaves, petioles and pseudostems of infected plants (Magee, 1927). Early infection results in complete yield loss as the affected plants do not produce fruit. In Australia, bunchy top almost caused the demise of the banana industry in the 1920s. However, strict phytosanitary regulations were put in place which effectively curbed its spread leading to containment (Dale, 1987). Bunchy top can be controlled through the removal of diseased plants, strict quarantine regulations and the use of disease-free planting material (Robson *et al.*, 2006). However, eradicating the disease is difficult and could be aided by complementing these measures with host resistance strategies. Although natural resistance does not occur, recent findings have demonstrated the possibility of generating transgenic bananas resistant to BBTV through an RNAi-mediated strategy (Borth *et al.*, 2011; Shekhawat *et al.*, 2012). In two studies, bananas were transformed with different constructs of the viral Rep gene (mutated Rep sequence, antisense strand of Rep, inverted repeat of Rep sequence and hairpin (hp) RNAs leading to the generation of BBTV resistant lines (Borth *et al.*, 2011; Shekhawat *et al.*, 2012). However, these studies have made limited attempts to identify the most critical gene for durable resistance and did not investigate if such an approach would provide broad spectrum resistance against the different BBTV isolates causing bunchy top disease.

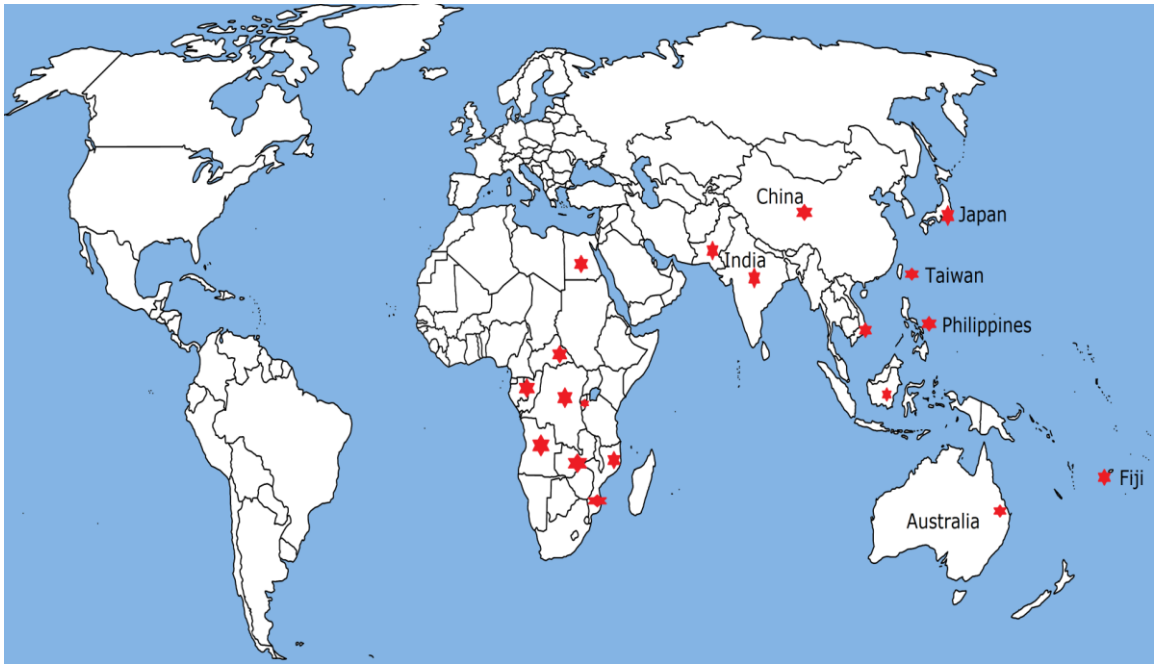


Figure 1.1: World map showing regional occurrence of banana bunchy top disease

1.5 Single-stranded plant DNA viruses

1.5.1 Geminiviridae

The *Geminiviridae* is a family of viruses possessing geminate virions approximately 18 nm x 30 nm (Fauquet, 2005) and genomes comprising monopartite or bipartite, circular single-stranded DNA (cssDNA) molecules (Gutierrez, 2000). The family derives its name from the distinct icosahedral capsid structure of its members (Gutierrez, 2000) and is generally categorised into 7 genera based on genome organisation, genome architecture (structure and organisation), type of vector and host range (Fauquet *et al.*, 2003; Varsani *et al.*, 2014). These include *Begomovirus* (mostly bipartite, infect dicots and vectored by whitefly), *Mastrevirus* (monopartite, infect monocots and vectored by leafhopper), *Curtovirus* (monopartite, infect dicots) and *Topocuvirus* (monopartite, infect tomato) (Fauquet *et al.*, 2003; Pilartz & Jeske, 2003) and the three newly identified genera comprising *Eragrovirus*, *Becurtovirus* and *Turncurtovirus* (Varsani *et al.*, 2014). The bipartite begomoviruses possess the most complex genome of these six groups, with two separately encapsidated DNA components designated -A and -B. The two components differ in nucleotide sequences but are similar in size and contain a common region (CR) highly conserved within a virus species, but distinct between virus species. The CR (200-250 bp) forms part of the intergenic region (IR) and contains an origin of replication and a promoter region.

DNA-A encodes the coat protein (CP) on the virion-sense strand while four proteins namely, Rep, TrAP (a transcriptional activation protein), REn (replication enhancer) and C4, are encoded on the complementary-sense strand. Component DNA-B encodes proteins associated with movement—BC1 and BV1—on the complementary-sense and virion-sense strands, respectively (Gutierrez, 1999). The multifunctional replication associated protein (Rep) regulates viral gene transcription, initiating as well as terminating virus replication (Hanley-Bowdoin *et al.*, 1999). The Rep is believed to function as an oligomer controlling gene expression through interaction with host proteins required in developmental and cell cycle regulation (Shepherd *et al.*, 2009). Geminiviruses replicate their circular ssDNA through a dsDNA intermediate by rolling-circle replication (RCR) (Gutierrez, 1999, 2000; Hanley-Bowdoin *et al.*, 1999; Jeske *et al.*, 2001) and also by a recombination-dependent replication strategy whereby incomplete short ssDNAs anneal to covalently-closed circular

DNA at homologous sequences, followed by elongation into dsDNA (Jeske *et al.*, 2001). In general, replication is initiated by the viral-encoded replication initiator protein (Rep), which creates a site-specific nick in the conserved nonanucleotide sequence TAATATT↓AC within the CR (Stanley, 1995).

Geminiviruses also encode proteins which bind plant host retinoblastoma-related (RBR) proteins (RBR is a negative regulator of cell cycle progression) (Gutzat *et al.*, 2012; Herwig & Strauss, 1997) to modulate host cell cycle DNA replication machinery. The mastrevirus-encoded RepA protein binds the RBR protein via an LXCXE motif, activating host DNA replication machinery necessary for viral DNA replication (Hanley-Bowdoin *et al.*, 2004).

1.5.2 Nanoviridae

The *Nanoviridae* is a second family of viruses which possess a cssDNA genome. Comprising two genera, *Nanovirus* (infecting legume plants) and *Babuvirus* (infecting banana plants), nanoviruses are distributed throughout Asia, Australia and Africa (Mandal, 2010). Nanovirus genomes consist of six to eight cssDNA molecules of about 1 kb each (Fauquet, 2005). Each DNA component contains at least one open reading frame (ORF) and at least one of the genome components of each virus encodes a Rep protein. Nanoviruses replicate in the nucleus of infected plants using RCR initiated by the viral encoded Rep, similar to the replication mechanism of the geminiviruses. The Rep proteins can be divided into two groups – the master Rep which is capable of trans-replicating the essential virus DNA components, and satellite Reps which are present in some virus isolates and are only capable of self-replication (Horser *et al.*, 2001). Other DNA components of nanovirus genomes include DNA-N (nuclear shuttle protein [NSP]), -M (movement protein [MP]), -S (capsid protein [CP]), -C (cell cycle link protein [Clink]) and DNA-U1 to –U4 (which may be pathogenicity determinants) (Timchenko *et al.*, 1999; Timchenko *et al.*, 2006). Conserved features of the nanovirus DNAs include a stem-loop common region (CR-SL) and a major common region (CR-M) (Burns *et al.*, 1995; Katul *et al.*, 1998). The CR-SL contains an invariant nonanucleotide sequence involved in Rep recognition which is also conserved amongst geminivirus genomes, while the CR-M contains promoter sequences and is the site of second-strand synthesis for replication (Burns *et al.*, 1995). The functions of the gene

products of BBTV DNA-M and DNA-N are comparable to those of the BC1 (MP and pathogenicity determinant) and BV1 (NSP)-encoded proteins, respectively, of the begomoviruses (Mandal, 2010). DNA-C contains a LXCXE motif with a retinoblastoma protein binding activity and modulates host DNA polymerase machinery to support viral replication (switching quiescent infected host cells to S phase capable of producing replication factors required by the virus) (Wanitchakorn *et al.*, 2000).

1.5.3 Banana bunchy top virus

BBTV, the type member of the genus *Babuvirus* and the causal agent of bunchy top disease, has a genome comprising at least six components (Fig.1.2). These include DNA-R (replication), -S (encapsidation), -C (cell cycle modulation), -M (cell to cell viral spread), -N (transporting DNA to and from nucleus) and -U3 whose function is yet to be determined (Burns *et al.*, 1995; Karan *et al.*, 1994; Karan *et al.*, 1997). All components encode a single ORF in the virion-sense with the exception of DNA-R which transcribes two mRNAs, one mapping to the major ORF (Rep) and the other to a smaller ORF within the major ORF (Beetham *et al.*, 1997). As with other nanoviruses, the master Rep is capable of trans-replicating the other DNA components (Horser *et al.*, 2001) and, as with geminiviruses, is critical in BBTV replication and viral DNA accumulation. The DNA-M protein (MP) has been implicated as a pathogenicity determinant and a silencing suppressor (Amin *et al.*, 2011) with a similar function to the BC1 gene of geminiviruses.

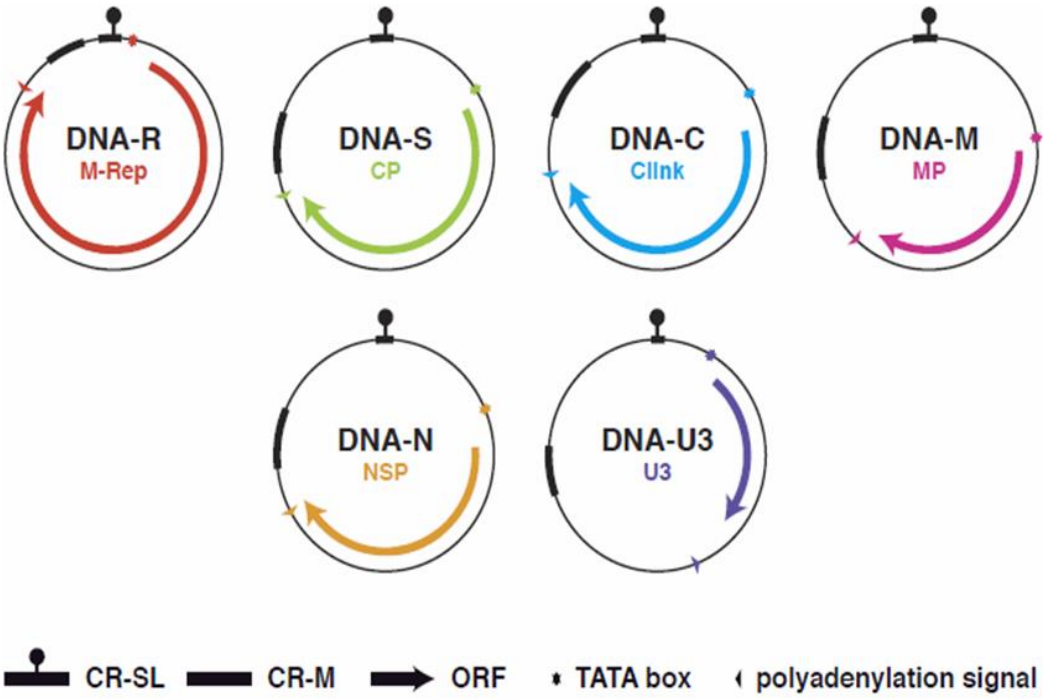


Figure 1.2: Six integral ssDNA genome components of *Banana bunchy top virus*

1.5.4 BBTV subgroups

Two BBTV subgroups have been reported based on nucleotide sequence analysis of DNA-R, -N and –S (Amin *et al.*, 2008; Bell *et al.*, 2002; Hu *et al.*, 2007; Karan *et al.*, 1994). These include the 'South Pacific' subgroup comprising isolates from Australia, the Pacific Islands, India, Iran, Myanmar, Pakistan and Africa (Egypt, Gabon, Burundi, Rwanda, Malawi, DRC, Cameroon) and the 'Asian' subgroup isolates from Philippines, Vietnam, China (including Taiwan) and Indonesia (Bananej *et al.*, 2007; Karan *et al.*, 1994; Kumar *et al.*, 2011; Wanitchakorn *et al.*, 2000). Based on analysis of full-length sequences of DNA-R, variability between isolates of the two subgroups was as high as 10%, while within isolates of each subgroup, the variability ranged from 1.9% in the South Pacific isolates to 3% in the Asian isolates. The nucleotide sequence of the CR-M is 96.6% conserved within South Pacific subgroup but only 68% between subgroups (Karan *et al.*, 1994). When the nucleotide sequences of DNA-N were used in the analyses, the nucleotide similarity between the two subgroups was 85.5%. Recently, an isolate from India with distinct nucleotide sequences has also been isolated and characterized (Banerjee *et al.*, 2014).

1.5.5 BBTV transmission

BBTV has a limited host range only within *Musa* sp (Dale, 1987). BBTV is persistently transmitted by the black banana aphid *Pentalonia nigronervosa (Hemiptera: Aphididae)* in a circulative, non-propagative manner (Hafner *et al.*, 1995; Harding *et al.*, 1991). Long distance spread is mainly through the movement of infected banana suckers and other planting materials (Allen, 1987), while mechanical transmission has not been successful (Thomas *et al.,* 2003). Vector transmission of BBTV is dependent on several factors including temperature, vector life stage and a minimum plant access period (Anhalt & Almeida, 2008). Transmission of the virus is favoured at temperatures of 25°C and 30°C compared with 20°C (Almeida *et al.*, 2009; Anhalt & Almeida, 2008).

1.6 Pathogen-derived resistance (PDR) in plants

The hypothesis that the use of a pathogen's own nucleic acid can be used to protect plants against invasion by the same pathogen was first reported in 1985 by Sanford and Johnston (1985). Theories on PDR propose that if sequences encoding critical functions required for infection are over-expressed within the host plant then the pathogenic process would break

down (Lindbo & Dougherty, 2005). In this regard, the expression of key viral proteins such as the coat protein, movement proteins or replication proteins can disrupt the infection process and so confer resistance (Abel *et al.*, 1986; Baulcombe, 1996; Cooper *et al.*, 1995; Grumet *et al.*, 1987). In two studies targeting a tobamovirus, expression of the *Tobacco mosaic virus* (TMV) CP gene was shown to confer resistance against TMV infection, with a high level of resistance observed in transgenic plants which expressed high levels of TMV CP (Abel *et al.*, 1986; Baulcombe, 1996). In this case resistance was proposed to result from the inhibition of virus disassembly (Register & Beachy, 1988). Similarly, the expression of MP genes have been shown to confer resistance to potex-, cucumo-, tobra- and tobamoviruses (Cooper *et al.*, 1995). In geminiviruses, several viral sequences were assessed for their potential to confer resistance to begomoviruses upon expression in transgenic plants, including defective interfering DNA components (Stanley *et al.*, 1990), the replication-associated protein (Bejarano & Lichtenstein, 1994; Bendahmane & Gronenborn, 1997; Day *et al.*, 1991; Hong & Stanley, 1996; Noris *et al.*, 1996; Sangaré *et al.*, 1999; Stanley *et al.*, 1990), the BC1 movement protein (Duan *et al.*, 1997; Duan *et al.*, 1997) and the coat protein (Kunik *et al.*, 1994). The transgenic plants resulting from these strategies showed delayed, attenuated symptoms with no durable resistance when infected with the geminivirus from which the transgene was derived. Abnormal phenotypes were also observed in certain cases (Hou *et al.*, 2000).

An alternative option to generate resistance to virus infection has involved the substitution in the amino acid sequence of Rep proteins to generate mutations in the most conserved amino acids (Rouhibakhsh *et al.*, 2011). The expression of mutant Rep sequences in plants has been shown to disrupt accumulation of viral DNA, attenuate viral DNA replication and slow symptom development (Rouhibakhsh *et al.*, 2011). However, expression of mutant Rep sequences may also enhance accumulation of viral DNA in some instances. For example, double mutagenesis of the AV2 gene of *Mung bean yellow mosaic India virus* (MYMIV) resulted in a 50-fold increase in the accumulation of viral DNA (Rouhibakhsh *et al.*, 2011).

In bananas, attempts to use mutated Reps to develop BBTV resistance have resulted in reduced viral DNA accumulation (Tsao, 2008). However, resistant banana lines have not been generated making it necessary to evaluate alternative approaches.

1.6.1 RNA interference (RNAi)

1.6.1.1 RNAi history

Small RNAs remained unknown until the 1980s when scientists interested in transgene technologies and plant–virus interactions described the concept of post-transcriptional gene silencing (PTGS). A number of attempts were made to provide an explanation for the phenomenon. By late 1980s, 'antisense RNA inhibition', a mechanism whereby an antisense RNA depresses or stops the expression of homologous endogenous genes was established in plants. Ecker and Davis (1986) transiently expressed sense and antisense constructs of the chloramphenicol acetyltransferase gene in protoplasts, and revealed that antisense constructs effectively suppressed the expression of the complementary sequences. Rothstein *et al.* (1987) demonstrated the same phenomenon in stably transformed plants, in which an antisense transgene was able to suppress the expression of a homologous sense transgene. The antisense technology was later found to be effective in the suppression of endogenous genes in stably transformed plants (Smith *et al.*, 1988; van der Krol *et al.*, 1988).

In the mid-1990s, a natural process in which RNA accumulation is suppressed in a sequence-specific manner, by homologous sequences was discovered in the nematode *Caenorhabditis elegans* by Guo and Kempues (1995). This process commonly referred to as RNA interference (RNAi) was also demonstrated in fungi, other animals/insects and plants (Mahmood ur *et al.*, 2008). RNAi is activated by the presence of double stranded RNA (dsRNA) molecules triggering specific destruction of homologous RNA sequences (Mahmood ur *et al.*, 2008; Zhang *et al.*, 2014). It is known to be involved in the regulation of developmental processes and is also a defence mechanism against viruses, transposons and other foreign nucleic acids (Duan *et al.*, 2008; Kasschau & Carrington, 1998; Ramesh *et al.*, 2007; Tabara *et al.*, 1998).

RNAi initiator dsRNA molecules may be endogenously derived, for example expressed from transgenic hairpin RNA (hpRNA) cassettes stably integrated into the plant genome, or exogenously derived, for example during virus infection (Singh *et al.*, 2014; Zhang *et al.*, 2014). Upon entry in the host cells, the dsRNAs are processed by a ribonuclease III-like enzyme called Dicer (Hannon, 2002) into 21-26 nt short interfering (si) RNAs (Fig. 1.3). The

siRNAs are then loaded into the RNA-induced silencing complex (RISC) and serve as a guide to direct Argonaute proteins in degrading homologous RNA molecules in the cytoplasm, or by annealing to homologous viral mRNA thereby curtailing translation and arresting viral protein synthesis (Incarbone & Dunoyer, 2013; Jagtap *et al.*, 2011; Wieczorek & Obrępalska-Stęplowska, 2014).

1.6.1.2 RNAi mediated virus resistance

RNAi has been utilised in the generation of transgenic plant virus resistance (Tohidfar & Khosravi, 2015; Younis *et al.*, 2014; Zhang *et al.*, 2014). In this regard, the use of inverted-repeat constructs containing sequences in both sense and antisense orientation separated by a small spacer region, expressed as hairpin RNAs, has been shown to induce effective gene silencing (Wesley *et al.*, 2001; Younis *et al.*, 2014; Zhang *et al.*, 2014).

The application of this strategy has resulted in genetically modified plants with resistance against many plant viruses, including potyviruses, ipomoviruses and geminiviruses (Baulcombe, 2004; Chen *et al.*, 2004; Kreuze *et al.*, 2008; Missiou *et al.*, 2004; Ramesh *et al.*, 2007; Yadav *et al.*, 2011). Intron-hairpin constructs producing large amounts of siRNAs are reported to elicit broad, robust and effective virus resistance (Di Nicola *et al.*, 2014; Zhang *et al.*, 2014). In the work by Zhang *et al.* (2014) and Di Nicola *et al.* (2014), the transgenic lines were resistant to infection by even distantly related (28% nucleotide variation) virus isolates

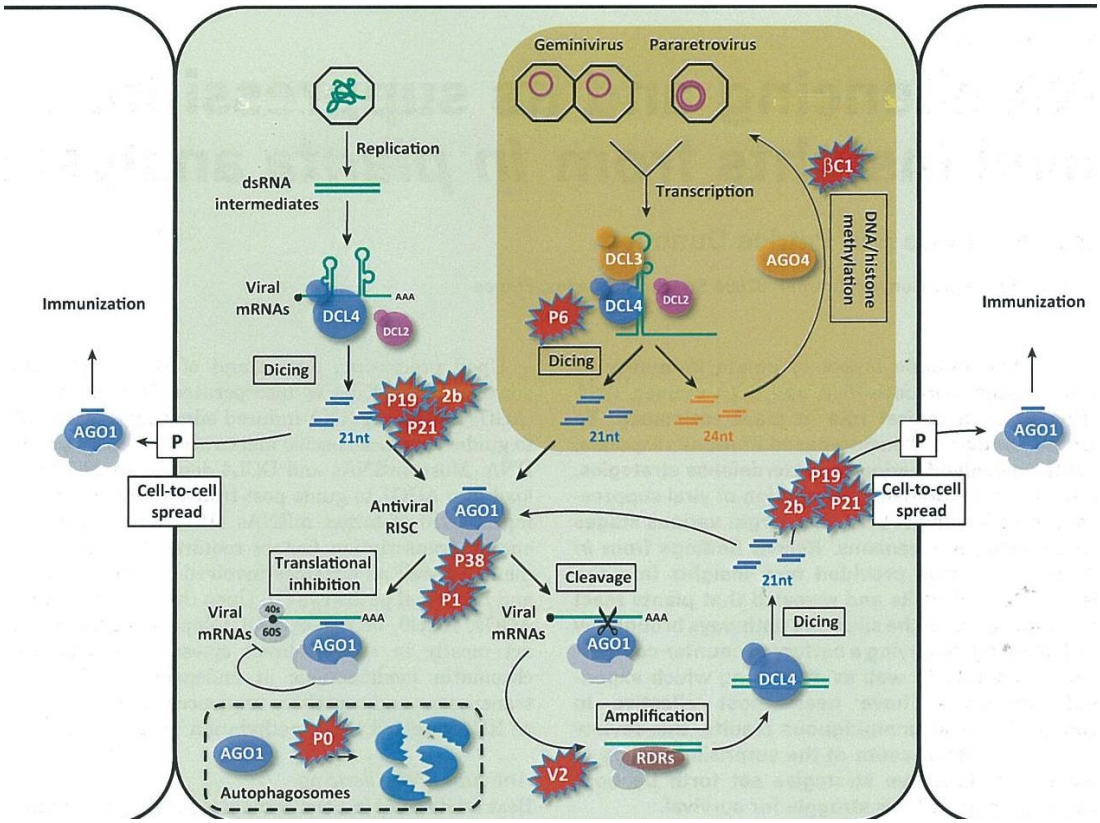


Figure 1.3: Diagrammatic representation of RNAi initiated by viral dsRNA or hairpin dsRNA (Incarbone & Dunoyer, 2013). Double-stranded RNA (dsRNA) and replication intermediates activate antiviral RNA silencing. The dsRNAs are processed into virus-derived small RNAs (vsRNAs) by RNaseIII-like enzymes known as Dicer-like proteins (DCL4, DCL3, and DCL2). The vsRNAs are incorporated into Argonaute (AGO)-containing RNA-induced silencing complexes (RISCs) leading to repression of translational inhibition and/or cleavage of viral RNA. Sliced viral RNAs are amplified by RNA-dependent RNA polymerases (RDRs) to produce more dsRNA substrates DCL processing. vsRNAs spread to adjacent cells via plasmodesmata inducing antiviral silencing response. Viral suppressors (P19, P21, P38, P1, 2b, P0, P6, V2, and bC1) are depicted in red at different stages repression of RNA silencing. P - plasmodesmata.

illustrating the effectiveness of intron-hairpin mediated virus resistance. RNAi gene constructs containing the beta satellite DNA gene (βC1; a pathogenicity determinant) associated with an Australian isolate of TLCV was reported to confer complete resistance in tobacco plants (Pakniat-Jahromy *et al.*, 2010). Additionally, Rep gene hpRNA constructs delivered complete resistance to *African cassava mosaic virus* (ACMV) as well as protection against heterologous cassava-infecting geminiviruses (Chellappan *et al.*, 2004). In most of these examples, a direct positive correlation between the level of RNA-based resistance and the accumulation level of the transgene siRNA was observed (Bucher *et al.*, 2006; Chen *et al.*, 2004; Kalantidis *et al.*, 2002). Recently, studies have reported the development of BBTV-resistant banana plants using an approach based on the RNAi pathway (Borth *et al.*, 2011; Shekhawat *et al.*, 2012). In these studies, BBTV DNA-R (encoding the master replication protein) was successfully used as the target for resistance through RNAi. In addition to DNA-R, gene products encoded by other DNA components also play pivotal roles in the life cycle of BBTV. In particular, the proteins encoded by DNA-M and DNA-S have been reported as silencing suppressors (Amin *et al.*, 2011; Niu *et al.*, 2009), and may be involved in modulating the hosts RNAi response. To date, these genes as well as those on DNA-N and –C have not been assessed for pathogen derived resistance through RNAi in banana.

Though many of the studies cited above demonstrate RNAi-mediated resistance against individual viral infections, there are few reports of the broad range effectiveness of this strategy to different virus species/isolates (Chellappan *et al.*, 2004), thus highlighting a gap of knowledge to further understanding the broad spectrum nature or extent of RNA silencing.

1.6.1.3 Broad spectrum resistance by RNAi

Plant virus isolates or species can cause simultaneous infection in the same host. Controlling such infections require a strategy or a combination of strategies that can knock out the multiple infections simultaneously. In addition, plant viruses encode multiple genes with specific integral functions. A knock out approach targeting multiple genes/components is likely to increase the effectiveness or durability of the resistance against the target pathogen. In the RNAi strategy, gene constructs made from conserved nucleotide regions against plant viruses have been shown to confer resistance against multiple isolates within

species. In addition, combining multiple target sequences derived from different virus species within one RNAi construct can result in effective silencing against multiple viruses (Chellappan *et al.*, 2004; Zhang *et al.*, 2011). Therefore, when designing RNAi constructs for broad spectrum resistance, specific requirements have been proposed which include targeting proteins with a critical function in the viral life cycle (Chellappan *et al.*, 2004), targeting highly conserved sequences (Gaba *et al.*, 2010; Vanitharani *et al.*, 2003) and achieving high levels of expression of the hpRNA (Vanderschuren *et al.*, 2009; Voinnet *et al.*, 1998). To maintain resistance against Potato virus Y, for example, a 93.9% nucleotide sequence homology with the transgene was required. However, the transgenic resistance failed against viral strains bearing 86.4% sequence similarity compared to the transgene (Gaba *et al.*, 2010). In this example, a 13.4% nucleotide sequence variation between transgene and viral strain led to resistance break down highlighting the specificity of RNAi resistance.

1.6.1.4 Silencing suppression

To overcome or withstand gene silencing mechanisms by host plants, many plant viruses encode proteins which function as suppressors of RNAi-mediated defences (Roth *et al.*, 2004; Voinnet *et al.*, 1998; Wieczorek & Obrępalska-Stęplowska, 2014). Such proteins are called viral suppressors of RNA silencing (VSR) or RNA silencing suppressors (RSSs). These proteins disrupt RNAi pathways at various stages of RNA silencing (Llave *et al.*, 2000; Meister & Tuschl, 2004) including sequestration, binding and stopping the systemic amplification of silencing factors such as siRNAs (Incarbone & Dunoyer, 2013).

Numerous plant viruses are known to encode one or several RSSs (Voinnet, 2005). The *Tomato bushy stunt virus* (TBSV)-encoded P19 suppresses RNA silencing in multiple plant species by sequestering siRNAs in a non-sequence-specific manner (Scholthof, 2006). P19 inhibits viral siRNAs from programming RISC by directly binding the siRNAs. Similarly, the begomovirus AC4 protein utilises base pairing to inhibit RISC from accessing the siRNA antisense strand as a guide for sequence-specific silencing. Silencing suppression is also exhibited by the interaction of the cucumoviral 2b gene product with Argonaute protein 1 (AGO1), curtailing the action of the RISC effector (Valli *et al.*, 2001). In BBTV infections, both the MP and Clink proteins are reported to function as silencing suppressors although their

modes of action have not been determined (Amin *et al.*, 2011). Disruption of methylation is another mechanism by which plant viruses counteract the host silencing mechanisms (Rodríguez-Negrete *et al.*, 2013). In their study, Rodríguez-Negrete *et al.* (2013) showed that the geminivirus Rep protein repressed the host maintenance DNA methytransferases. However, plants also possess proteins that can counteract the silencing suppression by viruses. For example, tobacco calmodulin-related protein has the ability to bind to dsRNA-binding domains of viral suppressor proteins namely HC-Pro (potyviruses) and 2b of the cucumber mosaic virus (Nakamura *et al.*, 2014).

1.6.1.5 Characterisation of RNAi in transgenic plants and natural infections

As a proof of the RNAi mechanism in transgenic or non-transgenic plants, profiling of small RNAs from hairpin gene constructs has been done using northern blotting or qRT-PCR (Zhao & Song, 2014). However, sRNA deep sequencing of plant-virus interactions is currently being applied to reveal the presence or up-regulation of certain microRNAs pre-virus or during virus infection in plants (Pradhan *et al.*, 2015; Wang *et al.*, 2014; Xiao *et al.*, 2014; Xu *et al.*, 2014). Characterisation of the nutrient composition of soybean plants transformed with inverted repeat constructs for multiple virus resistance and comparison with non-transgenic controls identified no significant biological changes, demonstrating that RNAi-mediated resistance did not lead to deleterious changes in this species (Zhang *et al.*, 2014).

1.7 Plant virus transmission and infection studies

Plant viruses are pathogenic nucleic acid complexes capable of causing infection in a specific range of host plants. Upon introduction into the plant, the virus hijacks the host's replication machinery to multiply its genome prior to spreading systemically from the point of entry. In order for the viral nucleic acids to move from plant to plant and perpetuate themselves in subsequent host generations, specialised insect vectors are often required (Drucker & Then, 2015). The insect vector acquires the virions over a specific feeding period and, upon relocation to another host, releases the virions to elicit a new infection. The establishment of a new infection relies on the acquisition and inoculation of the total genomic segments in their virulent state by the insect vector. Plant viruses encode genes that play unique roles in virus infectivity. For these viruses to induce infection artificially, an optimal mix of the different essential gene components are required to come together in specific host tissues

(Grigoras *et al.*, 2009). For artificial infection, cloned copies of virus genomes have been prepared to determine the minimum infective units (i.e. genes/components) and to fulfil Koch's postulates (Briddon *et al.*, 2001). Additionally, infectious clones provide a platform for plant-virus interaction studies such as the analysis of gene function by virus induced gene silencing (VIGS) (Baulcombe, 1999; Liang *et al.*, 2012) and protein/gene expression via plant-virus based expression vectors (Gleba *et al.*, 2007; Gleba *et al.*, 2004). In both cases, the gene of interest is fused to infectious nucleic acid copies of the viral vector before introduction into plant cells (Gleba *et al.*, 2007).

Genome segmentation in multipartite viruses creates a challenge for artificial infection since an optimal mix of the essential genomic components must enter an amenable cell type at the same time to induce infection. A copy of each of the segments is necessary for successful infection and reproduction of virions. The genome of BBTV comprises at least six single-stranded DNAs encapsidated individually and encoding specific genes for replication, movement, cell-cycle modulation, encapsidation as well as pathogenicity and transmission. It is plausible that for BBTV infection to occur most, if not all, of these components must be present in the same cell at the same time in an optimal ratio.

The development of infectious clones can involve a range of different techniques including PCR or rolling circle amplification (RCA), and cloning, followed by co-inoculation/biolistic bombardment into target cells/host plants (Malik *et al.*, 2011). Due to the circular nature of the DNA genomes of both geminiviruses and nanoviruses, RCA is an attractive method for the development of infectious clones and has been successfully demonstrated for several geminiviruses (Timchenko *et al.*, 1999; Timchenko *et al.*, 2006). In a study of *Cotton leaf curl virus* (CLCuV), a combination of DNA-A and a DNA-β satellite reproduced cotton leaf curl disease (Briddon *et al.*, 2001). Furthermore, the ability of βC1 to act as a suppressor of gene silencing was proven via a co-infection of infectious tandem repeat constructs of *Tomato leaf curl virus* (ToLCV) and the *Cotton leaf curl Multan virus* betasatellite (Eini *et al.*, 2012).

Despite the fact that BBTV DNA component sequences have been characterized and the functions of a number of their gene products determined, the minimum number of the genomic components required to reproduce bunchy top disease is not known. Moreover,

the function of DNA-U3 is yet to be determined though it is an integral component of the BBTV genome (Burns *et al.*, 1995). Recently, the infectivity of the nanovirus *Faba bean necrotic yellows virus* (FBNYV) was reported through the use of cloned DNA components of the FBNYV genome obtained using RCA (Timchenko *et al.*, 2006). In this study, at least five full-length cloned FBNYV DNA components (DNA-R, -C, -M, -U1, and U2) reproduced typical symptoms of yellowing and necrosis on inoculated plants. However, typical FBNYV virions were only observed in plants bombarded with eight cloned DNA components (DNA-R, -C, -M, -S, -N, -U1, -U2 and -U4). The study demonstrated that DNA-R, -M and -S were critical for infection, but that DNA-U1 and -U2 are also needed to reproduce typical symptoms (Timchenko *et al.*, 2006). In a more recent study of *Faba bean necrotic stunt virus* (FBNSV), eight components were individually prepared and agro-inoculated simultaneously in *Vicia faba* leading to infection (Sicard *et al.*, 2013). Although these two studies illustrate the possibility of generating artificial infections for multipartite nanoviruses, whether this artificial infection can be achieved for BBTV is yet to be explored.

1.8 Conclusions

In summary, geminiviruses and nanoviruses utilise similar replication mechanisms and show conserved functions in several key virus-encoded proteins. Resistance to geminivirus infection has been demonstrated using an RNAi approach. Transgenic resistance to nanoviruses has recently been reported with evidence of PDR against Hawaiian and Indian BBTV isolates using mutant Rep, partial and near full-length Rep (Borth *et al.*, 2011) and RNAi Rep gene constructs (Shekhawat *et al.*, 2012). The findings of Shekhawat *et al.* (2012) show that the mechanism of RNAi does indeed occur in banana and that this strategy could potentially be adapted to other BBTV gene targets as a means of generating resistance against BBTV.

Natural host-plant resistance remains the most desirable approach to controlling plant viruses. However, there are no known banana cultivars naturally resistant to BBTV and there are only a few successful examples where exclusion/quarantine combined with cultural controls has been used successfully to control the disease. As a result, bunchy top remains a limiting factor in banana production in many countries. Importantly, RNAi has been applied successfully in developing resistance to geminiviruses, a group of viruses that share many

molecular similarities to nanoviruses, especially in their modes of replication. Additionally some reports of RNAi-mediated resistance to BBTV have been reported.

This study aimed to further explore the potential of RNAi to generate BBTV resistance in bananas. Specifically, this research investigated which sequences of the multi-component genome of BBTV are the most effective for RNAi-mediated resistance and whether the BBTV-encoded silencing suppressor plays a major role in this RNAi resistance mechanism. This study also investigated the effectiveness of RNAi against heterologous BBTV isolates with the aim of generating broad spectrum resistance. As an alternative means of virus transmission, attempts were made to develop a BBTV infectious clone that could be used to artificially challenge the transgenic lines generated in this study. Finally, in an effort to explore natural BBTV resistance in *Musa* germplasm which may be exploited for production of new cisgenic and disease resistant banana varieties, 11 banana cultivars of diverse genotypes were infected with BBTV and monitored for disease symptoms.

CHAPTER 2

GENERAL METHODS AND MATERIALS

2.1 Introduction

This chapter outlines the general methods and materials used to achieve the aims and objectives. The first section describes general solutions used in nucleic acid amplification, cloning and sequencing, plant nucleic acid extraction, Southern and northern blot analyses. Details of embryogenic cell suspensions, a description of the binary plant expression vector and a list of constructs used in banana transformation are also provided. The second section details general methods applied to achieve the aims of the study. However, specialised methods and protocols used to achieve specific objectives are provided in their respective Chapters.

2.2 General solutions

2.2.1 Alkali lysis solution

Solution 1: GTE buffer (25 mM Tris-HCl, 10 mM EDTA, 50 mM glucose)

Solution 2: Lysis buffer (0.2 M sodium hydroxide, 1% SDS).

Solution 3: Neutralisation solution

2.2.2 Bacterial growth media and selection antibiotics

LB liquid media: 1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 170 mM sodium chloride (pH 7.0).

LB solid agar media: Liquid LB media prepared as above, with 1.5% bacto-agar added (Autoclave at 121° C for 15 min).

Ampicillin: Prepare a 100 mg/mL solution in deionized water, filter sterilise and store at -20°C.

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside): Prepare a 20 mg/mL (2% w/v) solution in dimethylformamide.

IPTG (Isopropyl-β-D-thioglactopyranoside): Prepare a solution by dissolving 0.2 g in 100 uL water (20% w/v), filter sterilize and store at -20° C.

Kanamycin (100 mg/mL): Dissolve 1 g of kanamycin in 10 mL of double distilled water then filter sterilize through a 0.22 μm filter.

Rifampicin (25 mg/mL): Dissolve 25 mg of rifampicin in 1 mL of dimethyl sulfoxide (DMSO) then filter sterilize through a 0.22 µm filter.

Super optimal broth (SOB): In 1 L of ddH_2O combine 5 g of yeast extract (0.5% w/v), 20 g of tryptone (2% w/v), 0.584 g of sodium chloride (10 mM), 0.186 g of potassium chloride (2.5 mM) and 2.4 g of magnesium sulfate (20 mM).

Super optimal broth with catabolite repression (SOC) medium: For 1 L combine 5 g of yeast extract (0.5% w/v), 20 g of tryptone (2% w/v), 0.584 g of sodium chloride (10 mM), 0.186 g of potassium chloride (2.5 mM), 2.4 g of magnesium sulfate (20 mM) and 200 g glucose (20% w/v).

PIPES (0.5 M): Dissolve 15.1 g of PIPES in 80 mL deionised water and adjust pH to 6.7 with potassium hydroxide or hydrochloric acid.

Inoue transformation buffer (TB): In 1 L of ddH_2O combine 10.88 g of manganese chloride (55 mM), 2.2 g of calcium chloride (15 mM), 18.65 g of potassium chloride (250 mM) and 20 mL of PIPES (0.02 M).

2.2.3 Gel electrophoresis buffers

Agarose gels: unless otherwise stated, gel electrophoresis analysis was done in a 1.5% agarose gel (1.5 g agarose dissolved in 100 mL of 1X TAE (1.5% w/v)) containing 0.025% (v/v) SYBR Safe-DNA gel stain (Thermo Fisher Scientific). TAE buffer used for RNA separation analysis was prepared in DEPC-treated water or deionized water.

Agarose gel loading dye (6X): 0.25 % (w/v) bromophenol blue dissolved in Tris-EDTA/glycerol (1:1).

TAE Buffer (10X): Tris-acetate-EDTA buffer was prepared using 48.4 g Tris base (tris (hydroxymethyl) aminomethane), 11.4 mL (17.4 M) glacial acetic acid and 3.7 g of EDTA-disodium salt dissolved in deionised water to a final volume of 1 L.

TBE Buffer (10X): Tris-borate-EDTA buffer was prepared by dissolving 108 g of Tris base, 55 g of boric acid and 4 mL of 0.5 M EDTA (pH 8.0) in deionised water to a final volume of 1 L.

2.2.4 Nucleic acid sequence clean up solutions

Prepare a solution of 3 M sodium acetate pH 5.2.

Prepare a solution of 125 mM EDTA pH 8.0.

2.2.5 Solutions used for plant nucleic acid extraction

$CHCl_3$: IAA: Chloroform-isoamyl alcohol constituted in a 24:1 ratio of chloroform and isoamyl alcohol, respectively.

CTAB buffer (DNA): DNA extraction buffer was composed of the following ingredients.

Table 2.1: CTAB buffer ingredients and quantities for 500 mL

Ingredient	Quantity
0.8% CTAB (cetyltrimethylethyl ammonium bromide) (0.8%)	4 g
1% N-lauroylsarcosine	5 g
220 mM Tris HCL	110 mL of 1 M stock
22 mM EDTA	22 mL of 0.5 M stock
0.8 M NaCl	23.38 g
0.14 M Mannitol	12.75 g
2% (w/v) PVP-10 (add in CTAB aliquot before use)	1 g in 50 mL
1.4% (v/v) 2-mercaptoethanol (add in CTAB)	14 µL/mL (700 µL in 50 mL)

CTAB buffer (RNA extraction buffer): 150 mM Tris base, 100 mM EDTA, 2% SDS (adjusted to pH 7.5 with concentrated HCl before adding SDS), add 1% 2-mercaptoethanol before use.

TE: Tris EDTA buffer comprising 10 mM Tris HCL, pH 8.0 and 1 mM EDTA.

2.2.6 Southern blot analysis buffers

Depurination buffer (0.2 M HCl): for 1 L, mix 11 mL concentrated HCl and 989 mL ddH_2O

Denaturation buffer (1.5 M NaCl and 0.5 M NaOH): prepare by mixing 87.66 g NaCl and 20 g of NaOH to 1 L of double distilled water and store at room temperature for up to 1 month.

Neutralization buffer: (1.5 M NaCl and 0.5 M) Tris-base mix 87.66 g and 60.50 g of each respectively to 1 L volume.

Detection buffer (0.1 M NaCl and 0.1 M Tris-base): Prepare by mixing 5.84 g and 12.11 g of each respectively to 1 L of ddH_2O.

20X SSC buffer (3 M NaCl and 0.1 M Tris-Sodium citrate): Prepare by mixing 350.6 g of NaCl and 176.4 g of Tris-Sodium citrate to 2 L of ddH_2O.

Low stringency buffer (2X SSC and 0.1% SDS): for 1 litre prepare by mixing 100 mL 20x SSC and 10 mL 10% SDS.

High stringency buffer (0.1X SSC and 0.1% SDS): for 1 litre add 5 mL 20X SSC and 10 mL 10% SDS.

Maleic acid buffer (0.1 M maleic acid and 0.15 M NaCl): Prepare by combining 23.214 g of maleic acid and 17.532 g of NaCl into 2 litres final volume of ddH_2O and pH to 7.5 with NaOH then autoclave at 121° C for 15 min.

Wash buffer (0.3%): Consist of maleic acid buffer supplemented with 0.3% Tween-20

Blocking solution (3% Skim milk powder in maleic acid buffer): This solution was made by dissolving 4.95 g of skim milk powder in 165 mL of freshly prepared maleic acid buffer on the day of washing.

Stripping buffer: 0.2 M NaOH and 0.1% SDS (w/v) prepared in a 1 L volume by mixing 8 g of NaOH and 10 mL of 10% SDS.

Other reagents included CDP star (Roche Life Science), Developer, Fixer and X-ray Film (Kodak).

2.2.7 Northern blot analysis buffers

RNA Extraction buffer: 150 mM Tris base, 100 mM EDTA, 2% SDS (adjusted to pH 7.5 with conc. HCl before adding SDS) and autoclaved at 121°C for 15 min, add 1% 2-mercaptoethanol just before use.

Other RNA extraction solutions: 5 M potassium acetate, chloroform-isoamylalcohol (49:1 v/v), 12 M $LiCl_2$, 70% and 100% ethanol (The salts were dissolved or reconstituted in DEPC-treated water).

Diethylpyrocarbonate (DEPC) treated water: 0.1% Diethylpyrocarbonate-treated deionised water was used in all RNA extraction and analyses i.e. 1 mL of DEPC was added to 999 ml deionised water and stirred overnight then autoclaved at 121°C for 20 min.

Hydrogen peroxide (3%): Gel casting trays, combs and apparatus were soaked in 3% hydrogen peroxide (i.e. reconstituted by mixing 200ml of 30% hydrogen peroxide into 1.8 L deionised water).

SDS: As an alternative to hydrogen peroxide, 10% SDS was sometimes used to clean all gel apparatus. Subsequently rinse the apparatus with nuclease-free water before performing RNA gel electrophoresis and blotting.

2.3 General Methods

General molecular experiments and studies were performed based on published protocols and procedures (Sambrook & Russell, 2012) and according to manufacturer's instructions specified in relevant sections. A detailed description is provided in specific chapters and sections where significant alterations were made from existing published protocols.

2.4 Nucleic acid amplification, cloning and sequencing

2.4.1 Oligonucleotides

Unless otherwise stated, oligonucleotides were designed using Vector NTI suite v10 (Invitrogen) based on published nucleotide sequences. Oligonucleotides were ordered from Gene Works Pty Ltd (Adelaide, South Australia). Prior to use, the oligonucleotides were reconstituted to 10 µM working concentration using nuclease-free water.

2.4.2 Polymerase chain reaction

The Expand High Fidelity PCR system (Roche Diagnostics) was used in PCR where subsequent cloning and vector construction was undertaken. The reaction mix comprised 10 mM dNTPs, 10 µM of each primer, 10 x Expand buffer (containing $MgCl_2$) and 2 units of enzyme mix, with reactions prepared to a final volume of 50 µL using nuclease-free water.

All standard PCR carried out for diagnostics/detection used the GoTaq® Green PCR Master Mix system (Promega) in which the reaction mix comprised the supplied PCR master premix (Taq DNA polymerase, dNTPs, $MgCl_2$ and reaction buffers), 10 ρmol of each primer and nuclease free water to a final volume of 20 µL.

In both systems, the PCRs were subjected to initial denaturation at 94°C for 2 min followed by 35 cycles at 94°C for 20 s annealing at various temperatures for 30 s and extension at 60°C for 1 min per kb, followed by a final extension step at 72°C for 2 min.

2.4.3 Reverse transcriptase PCR (RT-PCR)

All RT-PCR was carried out using the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) PCR kit from Promega and gene-specific reverse primers, as per the manufacturer's instructions. In summary, the reactions were prepared in two separate master mixes with first master mix comprising RNA template (2 µL being equivalent to approximately 0.5-1.0 µg), 10 ρmol of primer and nuclease-free water to a final volume of 10 µL. This mixture was incubated at 70°C for 5 min followed by chilling on ice for 2 min and then the addition of 10 µL of the second master mix. The second master mix contained 10 x M-MLV reaction buffer (4 µL), M-MLV transcriptase enzyme (0.5 µL), RNase inhibitor (0.25 µL), 10 mM dNTPs (1 µL) and 4.25 µL of nuclease-free water. The mixture was briefly vortexed and contents collected in the bottom of reaction tubes by brief centrifugation. Subsequently the mixture was incubated at 42°C for 1 h and then 70°C for 10 min. The resulting cDNA was subjected to second strand synthesis and PCR amplification as described in section 2.4.2.

2.4.4 Restriction enzyme DNA digestion

All restriction enzyme digests of plasmids (0.1-1 µg) or plant gDNA (5-20 µg) were performed in a 37°C water bath using appropriate buffers (New England Biolabs) for 2 h or overnight (at least 18 h).

2.4.5 Dephosphorylation of 5' ends of digested DNA

To prevent self-re-ligation, the phosphate groups at the 5' ends of endonuclease-digested plasmid DNAs were removed using calf intestinal alkaline phosphatase (Roche Life Science, Australia). The plasmid DNA from a restriction enzyme digest was incubated in a reaction mix containing 3 units of alkaline phosphatase, 1x alkaline phosphatase buffer and nuclease-free water to a final volume of 30 µL at 37°C for 1 h. After dephosphorylation, the reaction mix was incubated at 65°C for 10 min to deactivate the alkaline phosphatase followed by agarose gel separation and purification using PCR purification kit (Qiagen).

2.4.6 DNA ligation

Purified PCR products were ligated into pGEM-T Easy (Promega) as per the manufacturer's instructions. Ligation reactions for constructing vectors for plant transformation included

insert fragments and plasmid DNA generally in a 3:1 ratio. The plasmid DNA was digested and dephosphorylated as described previously. The respective DNA insert fragment was excised from the parent plasmid by specific restriction endonuclease and purified from agarose gel before ligation into the backbone plasmid. The ligation reaction mix consisted of 2 units of T4 DNA ligase (Promega), 1 x ligase buffer and plasmid/insert DNA. Ligations were incubated overnight at either 4°C for pGEM-T Easy or 14°C for other plasmid vectors.

2.4.7 Preparation of heat shock competent *Escherichia coli*

Escherichia coli XL1-Blue cells were used for cloning purposes in this study. The cells were prepared following the protocol of Sambrook and Russell (2012).

2.4.8 Transformation of bacteria with recombinant plasmids

Escherichia coli

Heat shock competent cells (50 µL) were combined with overnight ligation mixtures (20 µL) and kept on ice for 15 min, followed by heat shock in a 42°C water bath for 90 s. The mixture was immediately chilled on ice for 2 min before the addition of 100 µL of LB broth and incubation at 37°C incubator while shaking at 200 rpm for 1 h. Following the 1 h incubation, the transformed *E.coli* culture was spread onto solid LB plates containing the appropriate antibiotic/s before overnight incubation at 37°C.

Agrobacterium tumefaciens

Agrobacterium tumefaciens AGL1 cells were kindly supplied by D. Catchpoole (CTCB). Two µL of miniprep purified plasmid DNA was combined with 60 µL of cells and the mixture placed into an ice-cold sterile electro-cuvette. Electroporation of the mixture was performed at 2800 V using an EC100-Electroporator (Sigma-Aldrich) followed by addition of 940 µL of SOC into the cuvette and subsequent mixing by pipetting. The transformed cells were transferred into a 1.5 mL microfuge tube and then incubated in a 28°C shaker at 200 rpm for 2 h. After the incubation, 50 µL of transformed cells was plated onto LB media containing Rifampicin (25 µg/mL) and kanamycin (100 µg/mL). The plates were incubated at 28°C for 3 days.

Bacterial growth in liquid cultures

E. coli was cultured overnight at 37°C in LB containing the appropriate antibiotic with shaking at 200 rpm. Similarly, *Agrobacterium tumefaciens* was cultured in LB medium containing 25 µg/mL rifampicin and 100 µg/mL kanamycin at 28°C with shaking at 200 rpm for 3 days.

2.4.9 Isolation of plasmid DNA

Plasmid DNA was isolated using either the protocol described by Sambrook and Russell (2012) or using the Wizard® Plus SV Minipreps DNA purification kit from Promega as per the manufacturer's instructions.

2.4.10 Confirmation of cloning

Specific restriction endonucleases were used in digestion to confirm the presence of cloned DNA fragments. Fragments cloned into pGEM-T Easy vector were screened by *Eco*RI restriction digest and the size of band compared with the expected size. In this case, a typical restriction reaction digest mix contained 2 µL of 10X restriction buffer H, 2 U of *Eco*RI (0.2 µL) enzyme (Roche) and about 5 µL (250ng) plasmid. However, in preparation of the fragments for subsequent ligations and preparation of multimer's, specific digests were performed in appropriate buffer conditions.

2.4.11 Preparation of bacterial glycerol stocks

After confirmation of plasmid vector-insert presence, 850 µL of the transformed *A. tumefaciens* cell culture for each construct was added to 150 µL of 100% glycerol and stored at -80°C.

2.4.12 DNA sequencing

Sequencing was performed using the M13 primers or gene-specific primers and the BigDye Terminator v3.1 sequencing system (Thermo Fisher). Reaction mixtures contained 2 µL of purified plasmid DNA, 5 µL of 5 x sequencing buffer and either universal M13 primers or sequence-specific primers. Thermal cycling was carried out as follows: denaturation at 95° C for 30 s, annealing at 50° C for 20 s and extension at 60°C for 4 min, repeated for 30 cycles. Sequencing reactions were precipitated by adding 2 µL of 3 M sodium acetate (pH 5.2) and 2

µL of 125 mM Na_2EDTA (pH 8.0), mixing briefly and then adding 50 µL of 100% ethanol. Samples were incubated at room temperature for 15 min then centrifuged at 18000 x g before decanting the supernatant and washing the pellet with 250 µL of 70% ethanol. The pellet was dried under vacuum for 10 min and samples were delivered to the QUT Molecular Genetic Research Facility for sequencing using the 3500 Genetic Analyser (Applied Biosystems™).

2.4.13 Electrophoresis

Agarose gels

Agarose gels for nucleic acid separation and visualization were prepared as 1.5% agarose in TAE buffer containing 0.5 x SYBR safe DNA gel stain (Invitrogen). Gels were electrophoresed at 90-100 V for 45 min and nucleic acids visualised using a Safe Imager™ blue light transilluminator (Invitrogen) and the Syngene Geldoc System (Syngene).

Isolation and purification of DNA from agarose gels

Clean sterile scalpel blades were used to excise DNA fragments which were subsequently purified using either the High Pure PCR purification system (Roche) or Ultra Clean DNA purification kit (MO BIO Laboratories, Inc.) as per the manufacturer's instructions.

Polyacrylamide gels

Polyacrylamide gels were prepared as 17% acrylamide in 0.5x TBE buffer prepared in DEPC-treated water and electrophoresed at 60 mA for 2 h.

2.5 Nucleic acid extraction from plant tissue

2.5.1 Total genomic DNA

The CTAB protocol by James *et al* (2011) was used to prepare small scale DNA preparations for general PCR detection. Approximately 0.4 g of leaf tissue was pulverised (aided by acid washed sand) using a pestle and mortar in 1.5 mL CTAB or subjected to tissue lysing and incubated at 65° C for 30 min in a water bath. The supernatant (750 µL) was extracted with an equal volume of chloroform:isoamyl alcohol (24:1) and following centrifugation the DNA was precipitated by adding an equal volume of isopropanol to the supernatant and incubating for 5 min at room temperature. The resulting pellet was washed with 70%

ethanol and dissolved in 50 µL of de-ionised water and stored at -20° C. The protocol and steps followed for large scale DNA extract output are outlined in section 2.6.2.

2.5.2 Total RNA

Total RNA extraction was done following a modified banana RNA extraction protocol optimised by CTCB researchers. RNases were removed from all solutions used for RNA extraction by DEPC treatment and RNase-free plastic ware was used throughout (Quantum Scientific). Approximately 100 mg of plant tissue was ground using a tissue lyser at 30 rpm for 15 s in cryovials containing lead shots and frozen in liquid nitrogen. Powdered tissue was resuspended in 1 mL of pre-warmed RNA extraction buffer (2.2.7) followed by centrifugation at 18000 x *g* for 5 min to remove insoluble particles. The resulting supernatant was precipitated by the addition of 88 µL of 5 M potassium acetate and 200 µL of absolute ethanol (vortexed for 1 min before centrifugation at 18000 x g for 5 min). The soluble proteins and other materials in the precipitated RNA were removed by three chloroform:isoamyl alcohol (49:1) extractions. The RNA in the final supernatant (500 µL) was precipitated by addition of 167 µL of 12 M LiCl, gentle mixing by inversion and incubation at -20°C overnight. Following overnight incubation, the mixture was centrifuged at 20,000 x *g* at 4°C for 45 min. The resulting pellet was washed with chilled 75% ethanol before vacuum drying, dissolving in nuclease-free water and storage at -20°C.

2.6 Southern analysis

2.6.1 Synthesis of digoxigenin (DIG) labelled DNA probes

PCR probe synthesis was done to incorporate DIG 11-dUTP (digoxygenin-11-2'–deoxy-uridine-5'-triphosphate; Roche) in all DNA probes used for hybridisation. The 10 x DIG mix (2 mM) contained 0.7 mM dTTP and 1.3 mM dUTP at 3:1 ratio together with 2 mM of each dATP, dCTP and dGTP (Roche) subjected to PCR (section 2.4.2). The DIG-labelled PCR products were directly purified using UltraClean GelSpin DNA Extraction kit (M BIO labs, Inc.) after electrophoresing a small aliquot in agarose gel and determining the size differences between the labelled and non-labelled PCR products. Purified probes were eluted in a 50 µL final volume and stored at -20°C until required for hybridisation. Prior to hybridisation, the probe was prepared by dilution of 10 µL in a final volume of 50 µL with nuclease-free water followed by denaturation at 100° C for 5 min and chilling on ice for 5 min.

2.6.2 CTAB DNA extraction

For large scale genomic DNA (gDNA) extraction (i.e. to prepare DNA for Southern blotting), 1.5 g of banana leaf tissue was placed into cryovials containing lead shots and frozen in liquid nitrogen. A tissue lyser was used to grind the samples. The samples were processed 3 times at 30 Hz for 15 s each with sample tubes frozen in liquid nitrogen between each round.

Following grinding by tissue lyser, 1 mL of pre-warmed (65° C) CTAB buffer (2.2.5) was added to each sample and tubes incubated at 65°C for 30 min with shaking every 7 min. The samples were then centrifuged in a bench top centrifuge at 18000 x g for 1 min and 800 µL of supernatant transferred to sterile 2 mL tubes. The samples were extracted using an equal volume of chloroform:isoamyl and contaminating RNA removed by digestion with RNaseH (1 µg/mL) at 37°C for 1 h. DNA precipitation was done using equal volume of isopropanol followed by centrifugation for 10 min at 18000 x g. The DNA pellet was washed using ice-cold 70% ethanol by briefly vortexing and the tubes again centrifuged at 18000 x g for 5 min. The ethanol was removed using a pipette and the pellets dried under vacuum for 10 min before resuspending in 100 µL of nuclease-free water. The purity of the DNA preparations was determined by measuring the A_{260}/A_{280} ratios on a spectrophotometer and also by agarose gel electrophoresis.

2.6.3 gDNA restriction enzyme digestion for Southern blot analysis

All restriction enzyme digests of plant gDNA (5-20 µg) for Southern blot analysis were performed in a 37°C water bath using appropriate restriction endonucleases (BamHI or HindIII) and the recommended buffers overnight (at least 18 h). A typical digest reaction contained 5 µL of 10 x buffer, 2-15 U of high fidelity enzyme (NEB), 0.5 µL of 100 x BSA, 5-20 ug of DNA and nuclease-free water to a final volume of 50 µL.

2.6.4 Southern blotting

Following the overnight restriction enzyme digest, DNA was separated in agarose gels. Agarose gels for DNA separation were prepared to 0.8% in TAE buffer containing 0.5 x SYBR safe gel stain and electrophoresed at 65 V for 4 h. The agarose gel was photographed, rinsed with ddH_2O and soaked in depurination buffer for 10 min with gentle shaking. The gel was then rinsed with ddH_2O and incubated in denaturation buffer for 30 min with gentle

shaking, followed by rinsing again with ddH_2O and washing twice in neutralisation buffer for 30 min with gentle shaking. The DNA was then transferred to a nitrocellulose membrane (GE Healthcare Life Sciences) overnight (at least 18 h) in 20 x SSC buffer by the capillary method as described by Southern (1975).

Following the overnight transfer of DNA, the membrane was washed twice in 2 x SSC buffer for 2 min each time to remove the salt residues. The DNA was then fixed by either baking the membrane in an oven at 80°C for 2 h or ultra-violet immobilisation cross-linking (UV-cross-Linker) at 1200 x 100 mJ/cm^2. The nitrocellulose membrane was pre-hybridized by rotation in a roller bottle using 14 mL of pre-warmed DIG Easy Hyb Solution (Roche) for 1 h in a 42°C oven (Hybaid). The 50 µL reconstituted DIG-labelled PCR probe prepared in section 2.6.1 was added to 10 mL of pre-warmed DIG Easy Hyb Solution and used for overnight hybridisation of the nitrocellulose membrane with rotation in the 42°C oven.

After the overnight hybridisation, the membrane was subjected to several washes. Initially, the membrane was washed two times in 100 mL of low stringency buffer for 5 min with shaking. Following this, the membrane was washed two times in high stringency buffer in a 68°C oven using 150 mL of pre-warmed buffer for 15 min each time. Thereafter, the membrane was rinsed briefly in freshly made maleic acid washing buffer (with tween 20) for 5 min, prior to blocking in blocking solution for 30 min. The membrane was then incubated in blocking solution with a 1:20,000 dilution of anti-digoxigenin-antibody Products (AP) (mouse-derived DIG antibody) for 30 min at room temperature. To remove unbound antibody, the membrane was placed into wash buffer and incubated with gentle shaking for 15 min (twice) and then placed into detection buffer for 5 min. DNA hybridisation was visualized following the addition of CDP-star (10 µL in 990 µL of detection buffer) (Roche Life Sciences) onto the membrane and incubation in darkness for 5 min. The signals from the membrane were captured by exposure to x-ray film (AGFA) for between 20-150 min before processing in developer and fixer solutions for 2 and 5 min, respectively.

2.7 Northern blot analysis

2.7.1 Plasmid DNA preparation and sequencing

Plasmid DNA (5–10 µg) containing the gene of interest was digested overnight at 37°C with appropriate restriction enzymes, namely *Xma*I and *Bsr*GI, which cut the plasmid at 5' and 3' ends of the inserted gene fragment respectively to allow for T7 and SP6 transcription. An aliquot of digested plasmid DNA was analysed on a 1.5% agarose gel to confirm complete digestion. The linearised plasmid DNA was then gel purified using as described in section 3.3.2.2 and resuspended in nuclease-free water before storage at -20°C until needed for use. The purified linear plasmid DNA was then used as a template for riboprobe synthesis as detailed below.

2.7.2 Transcription and ^{32}P labelling of RNA probes

An RNA probe was prepared following a modified protocol described by Smith and Eamens (2012). Briefly, an *in-vitro* transcription reaction was set up using T7 or SP6 in separate reaction tubes. A mastermix for each probe template was prepared to contain final concentrations of 2.5 mM each of ATP, CTP, GTP (4 µL of pre-mix), 1.0 mM UTP (0.25 µL), 4 µL of 5x transcription buffer, 2 µL of 0.1 M DTT, 1 µL RNase inhibitor and 1 µL T7 RNA polymerase (SP6/T7). To this mixture was added 4 µL of ^{32}P-UTP followed by mixing and incubation at 37° C for 1 h. Following incubation, 1 µL of RNase-free DNase I was added to each sample followed by incubation at 37°C for a further 10 min to remove any residual DNA. Following the removal of the template DNA, 300 µL of carbonate buffer was added and incubated at 60°C to fragment the probe to an average size of ~50 nt. This was done as per the following formula: $t = (Li-Lf)/(k*Li*Lf)$ where:

t = time in minutes

Li = initial length of the probe in Kb

Lf = final length of the probe in kb (i.e. 0.05 if desired is 50 nt)

K is the rate constant= 0.11 Kb/min

Thereafter the reaction was stopped by addition of 20 µL of 3 M sodium acetate (NaOAC) and mixed by pipetting before use in hybridisation or stored at room temperature.

2.7.3 RNA hybridisation and signal detection

The membrane was placed RNA-side facing up in a hybridisation tube containing 50 mL of pre-warmed (42°C) sRNA hybridisation buffer before incubation at 42°C for 1 h. Thereafter, the RNA probe (section 2.7.2) was added directly to the pre-hybridized RNA-blotted membrane to hybridize overnight (16-20 h) at 42°C at a constant rotation in a hybridisation oven. After the overnight incubation, hybridisation buffer was discarded in ^{32}P radioactive waste disposal bottle and the membrane washed in 25 mL of pre-warmed (50°C overnight) 2× SSC/0.2% SDS for 20 min at 50°C with constant rotation in a hybridisation oven. The wash was repeated three times before transferring the nylon membrane to a heat-sealed plastic envelope and exposing on a K-Screen at room temperature. The K-Screen image was visualized using a Phosphor-Imager (GE Healthcare).

2.7.4 Polyacrylamide gel and electro-blotting RNA transfer

Polyacrylamide gels were made as 17% acrylamide in 0.5 x TBE buffer prepared in DEPC-treated water and electrophoresed at 60 mA for 2 h. Briefly, 11.25 mL of 40% acrylamide solution, 3 mL 10 x TBE buffer, 6.75 mL nuclease free water and 12.62 g of urea were mixed in a 50 mL sterile centrifuge tube resulting in approximately 30 mL total volume. The components were mixed by gentle inversion followed by incubation at 50°C for 20-30 min with occasional swirling until the urea completely dissolved. Immediately 15 µL of TEMED and 180 µL of 10% Ammonium persulfate (APS) were added to the solution and mixed by inversion each time. The solution was poured into a pre-assembled gel rig followed by insertion of the gel comb and allowed to set for 1 h at room temperature. The gel rig was transferred to an electrophoresis tank containing 0.5 x TBE buffer before removal of the gel comb and pre-electrophoresis at 100V for 30 min to warm the gel. Undissolved urea was flushed out from the wells using pipette tips and the buffer tipped out before adding a fresh buffer. Samples were loaded and gel electrophoresed at 100 V for 2 h prior to transfer of the gel to an electro-blotting apparatus.

The gel rig was carefully dismantled and RNA transfer set up by placing a pre-wetted positively charged nylon membrane (Hybond-N+; GE Healthcare Life Science) directly onto the gel, and subsequently covering both sides with Whatman filter paper and assembling in an electro-blotting apparatus (Thermo Fisher) containing 0.5 x TBE buffer. Following the

assemblage, RNA was transferred to the nylon membrane at 0.06 A for 100 min while stirring the buffer (ice block placed within the electro-blotting tank to inhibit RNA heat degradation during transfer) in a 4°C walk-in cold room. Following electro-blotting, the apparatus was disassembled and the nylon membrane kept wet in 0.5 x TBE buffer while transporting to the UV cross-linker. The membrane was cross-linked twice (one direction and 180° rotation) (auto-crosslink function) at 1200x100 mJ/cm^2. Following transfer, the membrane was either processed immediately (see below) or stored between Whatman filter paper in a sealed plastic bag at room temperature.

2.7.5 Stripping RNA membranes

When required, the RNA membrane was stripped of the previous probe by washing two times in heated 0.2% SDS in a hybridisation bottle with constant rotation at 50°C for 5 min.

2.8 Real time PCR (qPCR)

2.8.1 Extraction of genomic DNA

Genomic DNA was isolated following the CTAB-based method described in 3.2.1. The concentration of DNA in prepared extracts was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific) and then adjusted to 10 ng/µL and stored at −20°C. For quality verification, DNA was also electrophoresed in 1% agarose gels stained with 0.5x SYBR safe DNA gel stain.

2.8.2 Preparation of recombinant plasmid for standard curves

To prepare recombinant plasmids for standards, PCR was done to amplify the respective BBTV DNA component fragments followed by cloning and sequencing as described in section 2.4.10-2.4.12).

2.8.3 Plasmid DNA quantification by QuantiFluor dsDNA System for standard curves

Plasmid DNA quantification was performed using a QuantiFluor™ dsDNA System (Promega-E2670). The system contains a fluorescent double-stranded DNA-binding dye (504 nm Ex/531 nm Em) for sensitive quantitation of double-stranded DNA (dsDNA) as well as 20 x TE buffer and 50 x Lambda DNA standard for plasmid quantity estimation. In brief, the dye was thawed on ice while covered with aluminium foil to prevent exposure to light. In the

meantime, 20 x TE buffer was diluted to 1 x TE using nuclease-free water and the 200 x Quantifluor dsDNA dye to 1 x using TE buffer. The 50x Lambda DNA was re-constituted to 1 x in TE buffer to give a starting concentration of 2 ng/µL. A two-fold serial dilution of the standard Lambda DNA was then prepared in 1 x TE buffer.

The lambda DNA standard curve and test samples were aliquoted (100 µL) into triplicate wells in a 96 well optiplate. Quantifluor dsDNA dye (x 1; 100 µL) was added to each well and covered with aluminium foil to incubate at room temperature for 5 min. The optiplate was then read at 530/540 wavelength using a L550B luminescence spectrometer.

2.8.4 Calculation of plasmid copy number

The values obtained from the luminescence spectromete in 2.8.3 were exported to Microsoft Excel and a scatter plot was prepared and fitted with a linear regression trend line displaying linear equation and coefficient of correlation (R^2) based on RFUs (corrected using buffer-only controls) against the Lambda DNA standard curve. The linear regression equation was then used to calculate the concentration of the test samples. Plasmid copy number for qPCR was then calculated based on the following formula: Number of copies (copies/µL) = (total mass of plasmid (Calculated concentration (g/µL)/ (mass of 1 recombinant plasmid (n*1.096E-21); where n is the total base pairs of the recombinant plasmid and 1.096E-21 is the mass in grams per base pair (g/bp). The plasmids were dispensed in small aliquots sufficient for one serial standard and stored at -20°C until needed for qPCR.

2.8.5 Establishment of Standard Curves

Each of the purified plasmids prepared as described above (standard dilution series from 1.0×10^8 to 1.0×10^1 copies/µL with a 10-fold variation between steps) was used for standard curve preparation. The serial dilutions were re-suspended and mixed well by pipetting 20 times. Real time qPCR (rt-qPCR) was performed using 10 ng/µL (working concentration) of DNA and water as non-template control (NTC), with three replicates. The resulting cycle threshold values from the reaction were used to establish standard curves by plotting the threshold cycle (Ct) on the Y-axis and the natural log of concentration (copies/µL) on the x-axis. Linear regression equation $y = k x + b$ and coefficient of correlation (R^2) were displayed on the standard curve. Following the optimisation of the plasmid standard curve, qPCR

analysis of the unknown samples was set up including the plasmid as standard for determination of absolute and standard deviation from mean copy numbers.

2.8.6 Real-Time Quantitative PCR

All qPCR assays were performed on a Rotor-Gene Q Series Software 1.7 (2008 Corbett Life Science, Qiagen) in a 20 µL reaction volume containing 10 µL of 2 × SYBR Green PCR Master mix (Promega), 1 µL of diluted genomic DNA (10 ng) and 1 µL (3 and 5 µM) each of the gene-specific forward and reverse primers, respectively. Thereafter, all the quantifications were subjected to the following standard PCR reaction conditions: Initial hold at 50°C for 2 min; 95°C for 2 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, 72°C for 5 s and 82°C for 5 s. A final cycle of 95°C for 15 s, 60°C for 15 s, 95°C for 15 s was performed to provide dissociation curves (melting curves) for each sample, for determination of amplification specificity. All PCR reactions had three technical replicates for each biological sample tested and the experiment was repeated three times.

CHAPTER 3

GENERATION AND CHARACTERISATION OF TRANSGENIC BANANA EXPRESSING BBTV HAIRPIN RNA CONSTRUCTS

3.1 Introduction

RNA interference (RNAi) has been reported as an effective mechanism for generating virus resistance in a wide range of host-virus combinations, including viruses with both RNA and DNA genomes (Di Nicola *et al.*, 2014; Vu *et al.*, 2013; Zhang *et al.*, 2013). However, during natural infections many plant viruses express proteins which act to suppress RNAi to overcome or modulate the plant's defenses (Pumplin & Voinnet, 2013; Shimura & Pantaleo, 2011). RNAi-mediated resistance may therefore be enhanced if viral mRNAs encoding silencing suppressor proteins are targeted. In addition, with viruses containing multi-partite genomes the robustness of an RNAi-mediated strategy may be enhanced by targeting several different virus sequences (Zhang et al., 2011).

Although RNAi-mediated resistance targeting a range of virus sequences has been reported, not all target sequences result in robust resistance. For example, tomato plants expressing a RNAi construct targeting the AV1 gene (coat protein) of the begomovirus, *Tomato leaf curl* New Delhi *virus* (ToLCNDV), were shown to be resistant whereas those containing a construct targeting the overlapping region of AV1/AV2 (pre-coat protein) showed only a moderate level of tolerance (Vu et al., 2013). Since the sequence selected for design of RNAi constructs can clearly affect the level of resistance, assessing a variety of constructs which target different genes is important to improve the chance of generating resistance.

Recently, the development of BBTV-resistant banana plants using an approach based on the RNAi pathway has been reported (Borth *et al.*, 2011; Shekhawat *et al.*, 2012). In these studies, BBTV DNA-R was targeted for resistance as it encodes the master replication initiation protein (Rep), which plays an essential role in the BBTV life cycle. However, other proteins such as the movement protein (MP) encoded by DNA-M and the nuclear shuttle protein (NSP) encoded by DNA-N, also play central roles in the virus' life cycle. In addition to its role in viral DNA movement within infected cells, the MP also acts as a silencing suppressor (Amin et al., 2011; Niu et al., 2009) and so targeting this component may improve the success of an RNAi-mediated resistance strategy against BBTV. To date, the

genes encoded by DNA-M and –N have not been assessed as targets for developing BBTV resistance through RNAi.

Although limited to assessing the Rep gene as a target for RNAi, the study of Shekhawat *et al.* (2012) demonstrated that this approach may result in stable resistance against BBTV infection in bananas. Therefore, the aim of the research described in this chapter was to generate a suite of potential BBTV RNAi constructs, transform them into banana cells and regenerate banana plants for subsequent testing.

The specific objectives of this chapter were to:

1. Design and prepare RNAi gene constructs targeting BBTV DNA-R, -M and –N using sequences from both South Pacific and Asian subgroup isolates,
2. Transform banana embryogenic cell suspensions (ECS) with the RNAi gene constructs,
3. Regenerate and characterise transgenic banana lines.

3.2 Materials and Methods

3.2.1 BBTV samples, nucleic acid extraction and PCR

Dwarf Cavendish banana plants infected with a South Pacific (SP) isolate of BBTV were maintained at QUT. Leaf tissue from BBTV-infected banana plants collected in Vietnam (designated VN), representing an Asian subgroup isolate, was stored dried over silica gel at room temperature at QUT.

Three BBTV DNA components were chosen as targets for the preparation of RNAi constructs, namely DNA-R, -M and –N. Published sequences of SP subgroup isolates were retrieved from GenBank and separately aligned using the AlignX function of Vector NTI Advance suite v.10.3.0 (Invitrogen). The most conserved region within the ORF of each component was identified. To increase confidence that the selected sequences would generate siRNAs, analysis was carried out using the BLOCK-iT RNAi-Designer software package (Invitrogen). PCR primers were designed from the consensus sequences for amplification of the component-specific fragments and some of the primers were flanked by *Bam*HI and *Hind*III restriction sites to facilitate subsequent manipulation (Table 3.1). Total

nucleic acid (TNA) was extracted from BBTV-infected banana leaf tissue using a CTAB-based method in section 2.6.2. The nucleic acid was subsequently used as template for the amplification of fragments of BBTV DNA-R, -M and –N using PCR. PCR was carried out using the Expand High Fidelity PCR system (Roche) according to the manufacturer's instructions and the amplified DNA fragments were visualised following agarose gel electrophoresis. Amplicons of the expected size were extracted using Quantum Prep Freeze 'N Squeeze DNA extraction spin columns (Bio-Rad) and ligated into pGEM-T Easy (Promega) following the manufacturer's instructions.

3.2.2 Cloning and sequencing

Heat-shock competent *E. coli* were transformed and selected as described in section 2.4.8. Isolation of plasmid DNA from bacterial clones was done using the alkali-lysis plasmid mini-preparation protocol described by Sambrook and Russell (2001). To confirm the presence of cloned DNA fragments in plasmids, an aliquot was digested using two units of the respective restriction endonuclease and the presence of the expected size band visualised on agarose gels. To confirm the identity of cloned fragments, sequencing was done using the Big Dye Terminator mix v3.1 (Applied Biosystems) and universal M13 primers, and sequences were analysed using ContigExpress (Invitrogen). Several plant binary vectors, including pOPT-EBX, pNad-Ubi-Fea1, pGen-2 and pGemT-CCoAOMT used for the preparation of hairpin vectors were provided by QUT. The preparation of the hairpin vectors for plant transformation is described in detail in the results section below.

3.2.3 Transformation of Agrobacterium

Agrobacterium tumefaciens strain AGL1 cells were transformed by electroporation as described in section 2.4.8. Following PCR confirmation of the presence of the correct vector DNA, overnight bacterial cultures were grown as described in section 2.4.8 and the bacterial cells were pelleted by centrifuging at 1000 x *g* for 10 min. The pellet was re-suspended in 10 mL of BRM media containing 200 µM acetosyringone and incubated at room temperature on a shaker at 70 rpm for 3 h. The OD_{600} was then adjusted to 0.5 before being used for transformation of banana embryogenic cell suspensions (ECS).

Table 3.1: PCR primers for preparing binary vectors[a]

Amplicon name	Forward primer (5`-3`)	Reverse primer (5`-3`)	Amplicon length (bp)
Preparation of SP multimeric cassettes			
SP-Rep5`	GCGGCGCGATATGTGGTATGCTGG	GCggatccaagcttACCAAACTCGAAGGGACCTTCG	280
SP-RepIO	GCGCGTGAAACGCACAAAAGGCC	GCggatccaagcttGGAGAATAAAACGCATTTCTCG	279
SP-MP	GCggatccGGCATTAACAACAGAGCGGG	GCggatccTCCAGCGTTTCCTCGTCTTCC	340
SP-NSP	GCaagcttCGATCAGAGACGATGACGGAG	GCaagcttCTCCAATCTATTCCTGGCGC	151
Preparation of pOPT-EBX:Ubi-nosT			
nosT	GCgtcgacCGTTCAAACATTTGGCAATAAAGTT	GCaggcctGATCTAGTAACATAGATGACACCGCG	250
syntron	GCaggcctcccgggATATTtgtacaCTGCAAGAAAACAAAAT	GCaggcctgttaacATATTacgcgtCGTAAGATTTTTATTTT	102
SP BBTV hairpin sense primers			
SP-Rep 5`	GCcccgggGGCGCGATATGTGGTATGCTGG	GCtgtacaACCAAACTCGAAGGGACCTTCG	280
SP-RepIO	GCcccgggGCGTGAAACGCACAAAAGGCC	GCtgtacaGGAGAATAAAACGCATTTCTCG	279
SP-MP	GCcccgggGGCATTAACAACAGAGCGGG	GCtgtacaTCCAGCGTTTCCTCGTCTTCC	340
SP-NSP	GCcccgggCGATCAGAGACGATGACGGAG	GCtgtacaCTCCAATCTATTCCTGGCGC	151
SP BBTV hairpin anti-sense primers			
SP-Rep 5’	GCgttaacGGCGCGATATGTGGTATGCTGG	GCacgcgtACCAAACTCGAAGGGACCTTCG	280
SP-RepIO	GCgttaacGCGTGAAACGCACAAAAGGCC	GCacgcgtGGAGAATAAAACGCATTTCTCG	279
SP-MP	GCgttaacGGCATTAACAACAGAGCGGG	GCacgcgtTCCAGCGTTTCCTCGTCTTCC	340
SP-NSP	GCgttaacCGATCAGAGACGATGACGGAG	GCacgcgtCTCCAATCTATTCCTGGCGC	151
Asian BBTV hairpin sense primers			
VN-Rep	GCcccgggATGGTATATCAAGTGGAGAG	GCtgtacaTCAACTCTGCTTGCACTCTG	349
VN-MP	GCcccgggATGGCATTGACAACAGAGCG	GCtgtacaTGTATTAAAACATAGGGCCG	354
VN-NSP	GCcccgggGCAGAAGCGATGGATTGGGC	GCtgtacaCACTTCAGATCACATGATTC	440
Asian BBTV hairpin anti-sense primers			
VN-Rep	GCgttaacATGGTATATCAAGTGGAGAG	GCacgcgtTCAACTCTGCTTGCACTCTG	349
VN-MP	GCgttaacATGGCATTGACAACAGAGCG	GCacgcgtTGTATTAAAACATAGGGCCG	354
VN-NSP	GCgttaacGCAGAAGCGATGGATTGGGC	GCacgcgtCACTTCAGATCACATGATTC	440

[a]Restriction endonuclease sites used for cloning are in lower case font and underlined.

Rep- replication fragment, RepIO-Replication internal ORF fragment, MP-movement protein, NSP-nuclear shuttle protein and bp-base pairs.

3.2.4 *Agrobacterium* transformation of banana ECS

Two banana ECS cell lines (DC35-8 and DC6-5) derived from *Musa* sp. cv. Dwarf Cavendish (AAA-Cavendish subgroup), and two additional lines (GN263-8 and GN76) from cv. Grand Nain were prepared as described by Cote *et al*. (1996) and Dheda *et al*. (1991) and transformed as described by Khanna *et al*. (2004). The details of all the media used in the transformation are outlined in Appendix 1. Four days after routine subculture, the ECS were sieved through a 500 µm mesh into a sterile beaker. The cells were allowed to settle for 2 min before removing the supernatant by pipetting. ECS were transferred to sterile tubes and heat-shocked at 45°C in a water bath prior to transformation, then 1 mL of ECS was mixed with 5 mL of pre-induced (section 2.4.8) *A. tumefaciens*. Pluronic F68 (polyoxyethylene-polyoxypropylene block copolymer) was added to the ECS/*A. tumefaciens* mix at a final concentration of 0.02% (v/v) and the mixture inverted gently. The mixture was centrifuged at 1000 x *g* for 10 min in a benchtop centrifuge before a gentle mix by inversion and then allowing the cells to rest at 27°C for 30 min, followed by the removal of excess liquid from the settled cells by pipetting. ECS were then aspirated onto sterile 70 mm diameter filter papers previously placed onto co-cultivation media (CCM) in 90 mm Petri dishes and the ECS were incubated for 3 days at 22°C in the dark. As a transformation control, pART-Test7 (35S-gfp-nosT) was included with each transformation experiment.

3.2.5 Selection and regeneration of transgenic lines

Following incubation in the dark for three days, transient GFP expression was visualised using a fluorescence microscope in ECS transformed with the control construct to determine the efficiency of transformation. ECS were then washed six times using 50 mL BL media supplemented with 200 µg/mL timentin (GlaxoSmithKline, Australia) and then aspirated onto sterile filter papers on BL media containing 25 µg/mL kanamycin. The ECS were maintained in the dark at 25°C for two weeks and were then subcultured onto fresh BL media containing 50 µg/mL kanamycin. After another two weeks in the dark, ECS were subcultured onto fresh BL media containing 100 µg/mL kanamycin and were grown at 27°C for 8 weeks in the dark before being transferred to M3 media for embryo formation. The ECS were maintained on M3 media for 12 weeks with routine sub-culturing every 3 weeks before embryo germination was initiated by transfer of the ECS onto M4 media. The ECS were maintained on M4 for 12 weeks in the light with routine subculturing every 3 weeks.

During this 12 week period, individual shoots/plantlets were sequentially transferred to rooting (M5) media in separate growth containers and allowed to develop roots and shoots prior to molecular characterisation by PCR and Southern blot analysis.

3.2.6 PCR screening for confirmation of transgenes

Transgenic plants were screened for the presence of transgenes using PCR as described in section 2.4.2. Hairpin-specific forward primers were used in combination with either the syntron forward or reverse primers (in separate reaction mixes) to confirm the presence of both arms of each hairpin construct. PCR to check for *Agrobacterium* contamination was carried out using *vir*C operon-specific primers VCF (5' GCCTTAAAATCATTTGTAGCGACTTCG 3') and VCR (5' TCATCGCTAGCTCAAACCTGCTTCTG 3').

3.2.7 Southern blot analysis

Southern blotting was carried out as described in section 2.6.4. Total gDNA was isolated and purified as described in section 2.6.2. The gDNA was quantified using a Nanodrop instrument (ThermoFisher Scientific) and the quality was assessed by gel electrophoresis before restriction digest using BamHI. Primers which amplify the nptII sequence present in each construct were used to prepare a DIG-labelled probe as described in section 2.6.1.

3.2.8 RT-PCR

Total RNA was extracted from leaf tissue as described in section 2.5.2 and RNA was reverse transcribed into cDNA as described in section 2.4.3 using a nos-T reverse primer (5' CTGTTGAATTACGTTAAGCATG 3'). The resulting cDNA was subjected to PCR using a combination of sequence-specific forward primers and syntron forward or reverse primers (see below).

3.3 Results

3.3.1 Identification of target sequences for preparing the RNAi hairpin cassettes

Three BBTV DNA components were chosen as targets for the preparation of constructs, namely DNA-R, -M and –N. Published sequences of SP subgroup isolates were separately aligned and the most conserved region within the ORF of each component was identified. For DNA-R (which contains two ORFs), 11 isolates were aligned and two regions of 280 nt and 279 nt (both with 94.6% nucleotide identity) were selected and designated SP-Rep5` and SP-RepIO, respectively (Table 3.2). These two regions were chosen in an attempt to eliminate replication by silencing either the Rep-encoding major ORF sequence (Rep5') or both the Rep and the gene product encoded by the small DNA-R internal ORF (RepIO). For DNA-M, the ORF sequences from seven published isolates were aligned and a region of 340 nt with 92.4% sequence identity (designated SP-MP) was selected while for DNA-N, four published isolates were aligned and a 151 nt region (designated SP-NSP) with 98.7% sequence identity was chosen (Table 3.2). The start codon for each ORF was omitted from the selected sequences to ensure that a translatable RNA was not expressed from the hairpin cassettes.

Similarly, Asian subgroup DNA-R, -M & –N sequences were aligned and the most conserved region of each ORF was selected for designing constructs. For DNA-R, a region of 349 nt (designated VN-Rep) with 96% sequence identity was chosen while, for DNA-M and DNA-N, regions of 354 nt (VN-MP) and 440 nt (VN-NSP), respectively, were chosen with at least 93% sequence identity (Table 3.2).

To increase confidence in the selected sequences for generating successful siRNAs, they were analysed using the BLOCK-iT RNAi-Designer software package to predict *in silico* the capacity of selected sequences to generate siRNAs. The BLOCK-iT RNAi-Designer identifies regions of the selected sequence with a high-probability of successful target knockdown and categorises these accordingly. Following analysis a population of putative siRNAs ranging from 18-21 bp in length was generated from each of the selected sequences and each population included putative siRNAs rated 'greatest likelihood', 'very likely' and 'likely' to result in greater than 70% knock-down of their respective target. Based on this analysis, the selected sequences were considered to be suitable for preparing the hairpin constructs.

Table 3.2: Selected BBTV sequences used for the preparation of RNAi constructs

Amplicon name	No. of isolates aligned	Fragment length (bp)	Sequence identity (%)	Location
SP- Rep5`	11	280	94.6	131-410
SP-RepIO	11	279	94.6	461-738
SP-MP	7	340	92.4	281-620
SP-NSP	4	151	98.7	498-648
VN-Rep	3	349	96	185-533
VN-MP	2	354	93	309-661[a]
VN-NSP	2	440	93	286-725 [a]

[a] The selected region for this target was identified only from Taiwan isolate DNA sequences available in the NCBI database at the time.

3.3.2 Construction of BBTV silencing cassettes

DNA was extracted from leaf tissue collected from a BBTV-infected banana plant growing in south-east Queensland (SP subgroup) and Vietnam (VN subgroup). PCR was carried out using primers designed to amplify the target regions of DNA-R, -M and –N (Table 3.2). Amplicons of the expected size were obtained in each case and these were cloned into pGemT-Easy and sequenced to confirm their identity. A total of four silencing cassettes based on SP BBTV sequences (Rep5', RepIO, MP and NSP; Fig. 3.1) and three cassettes based on VN BBTV sequences (Rep, MP and NSP) were generated using this approach.

An additional six multimeric SP BBTV silencing cassettes, containing either two or three of the individual SP BBTV component sequences in tandem, were also constructed. To generate these cassettes, pGemT-Easy clones containing the two different SP DNA-R sequences (Rep5' and RepIO) were linearised using appropriate restriction enzymes in separate reactions. DNA-M and DNA-N fragments were then excised from pGemT-Easy clones using the appropriate restriction enzyme and these were subsequently ligated into the linearised DNA-R clones to generate the four constructs designated Rep5`MP, Rep5`NSP, RepIOMP and RepIONSP (Fig 3.1). To complete the final two multimeric constructs, a *Hind*III-digested DNA-N fragment was ligated into *Hind*III-linearised pGemT-Rep5'MP to generate pGemT-Rep5'NSPMP, while a *Bam*HI-digested DNA-M fragment was ligated into *Bam*HI-linearised RepIONSP to generate pGemT-RepIONSPMP (Fig 3.1). The integrity of all clones was verified by sequencing.

3.3.3 Preparation of hairpin constructs for plant transformation

The pOPT-EBX binary vector was used as the base vector for transformation of banana cells with the BBTV RNAi hairpin constructs. This vector consists of an NPTII expression cassette for selection of transformed plant cells with the antibiotic kanamycin and a multiple cloning site within the T-DNA region to allow for insertion of additional sequences. To ensure high level constitutive expression of the hairpin cassettes, pOPT-EBX was modified so that expression of the BBTV hairpin cassettes was controlled by the maize (*Zea mays*) polyubiquitin (Ubi) promoter and an *Agrobacterium tumefaciens* nopaline synthase terminator (nosT). The Ubi promoter was excised from the plasmid pNad-Ubi-Fea1 (kindly provided by Mr. Don Catchpoole, QUT) by double digestion with *Hind*III and *Bam*HI and

ligated into appropriately digested pOPT-EBX. Specific PCR primers flanked by *Sal*I and *Stu*I sites (Table 3.1) were used to amplify the nosT sequence from the pGen-2 vector (kindly provided by Mr. Don Catchpoole, QUT) and the amplicon was cloned into pGemT-Easy and sequenced. The nosT amplicon was then digested with *Sal*I and *Stu*I and ligated into appropriately digested pOPT-EBX containing Ubi.

To facilitate the bi-directional cloning required for hairpin RNA production, an adapter fragment comprising a 102 nt synthetic intron ('syntron') sequence was incorporated into the modified pOPT-EBX between Ubi and nosT. Primers flanked by *Stu*I restriction sites and incorporating *Xma*I/*Bsr*GI restriction sites (5` primer) or *Hpa*I/*Mlu*I restriction sites (3` primer) (Table 3.1) were designed and used to amplify the syntron sequence from the pGemT-CCoAOMT vector provided by Dr Patrick Bewg (QUT). The amplicon was ligated into pGemT-Easy and sequenced. Following digestion with *Stu*I, the syntron was ligated into *Sma*I-linearised pOPT-EBX:Ubi-nosT resulting in the base binary vector for preparing the BBTV hairpin constructs.

The 10 SP BBTV DNA silencing cassettes described previously (Fig 3.1) were re-amplified from the respective DNA clones using sequence-specific primers (Table 3.1) containing 5` *Xma*I and 3` *Bsr*GI restriction sites for hairpin sense fragments, or 5` *Hpa*I and 3` *Mlu*I restriction sites for hairpin antisense fragments. These were cloned and sequenced and sequential ligations were then used to insert firstly the sense arm (using *Xma*I and *Bsr*GI), followed by the antisense arm (using *Hpa*I and *Mlu*I), into the binary vector pOPT-EBX:Ubi-nosT. An example of a binary vector containing a BBTV SP Rep5' hairpin cassette is shown in Fig 3.2. Following cloning, the presence of the hairpin cassettes was verified by PCR (Fig. 3.3) using the specific forward primer for each cassette in combination with either the syntron forward or reverse primers.

In summary, a total of 13 binary vectors were prepared for transformation into banana ECS. Of these, 10 were designed to target the SP subgroup of BBTV isolates and three were designed to target the Asian subgroup of BBTV isolates (designated VN). The 10 SP binary vectors comprised four single gene target vectors and six multimeric gene vectors with the latter containing two or three of the individual BBTV component sequences in tandem

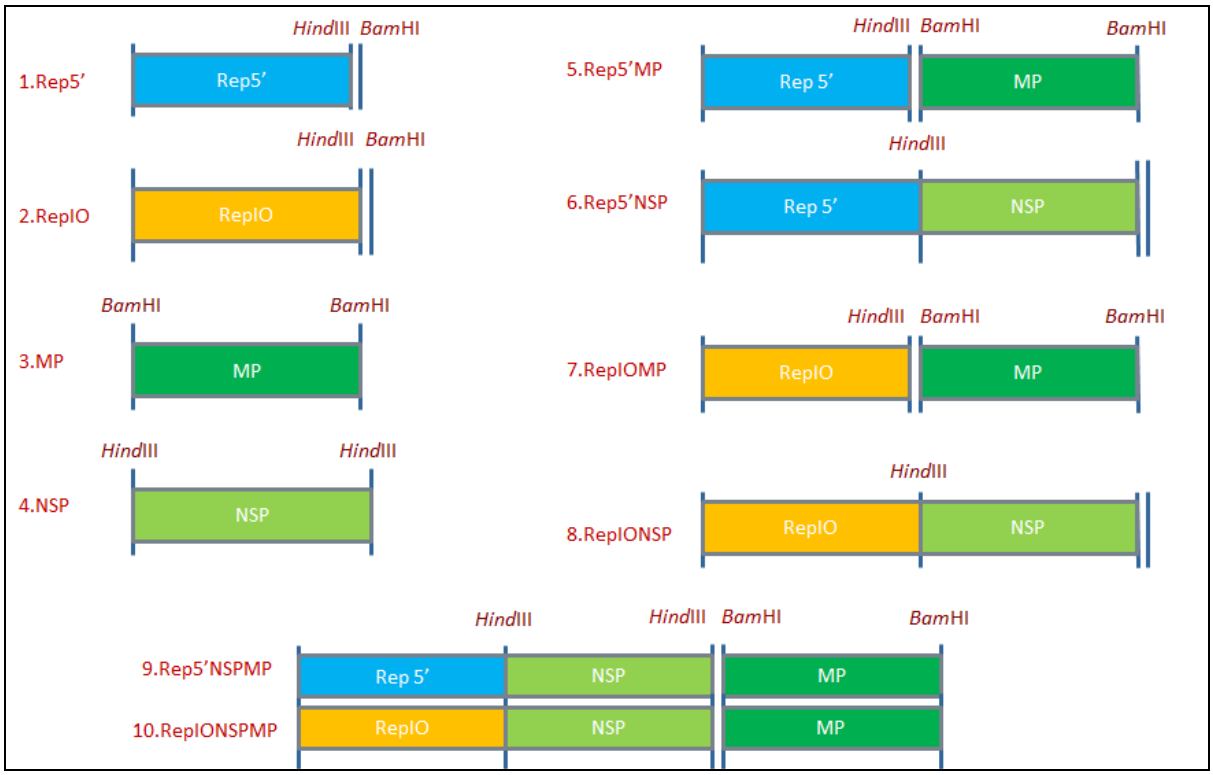


Figure 3.1: Schematic representations of the BBTV SP cassettes prepared in pGemT-Easy. Cassettes 1-4 contain a single BBTV target sequence and cassettes 5-10 contain multiple target sequences. Rep5': Replication fragment; RepIO: Replication internal open reading frame fragment; NSP: Nuclear shuttle protein target fragment; MP: Movement protein target fragment.

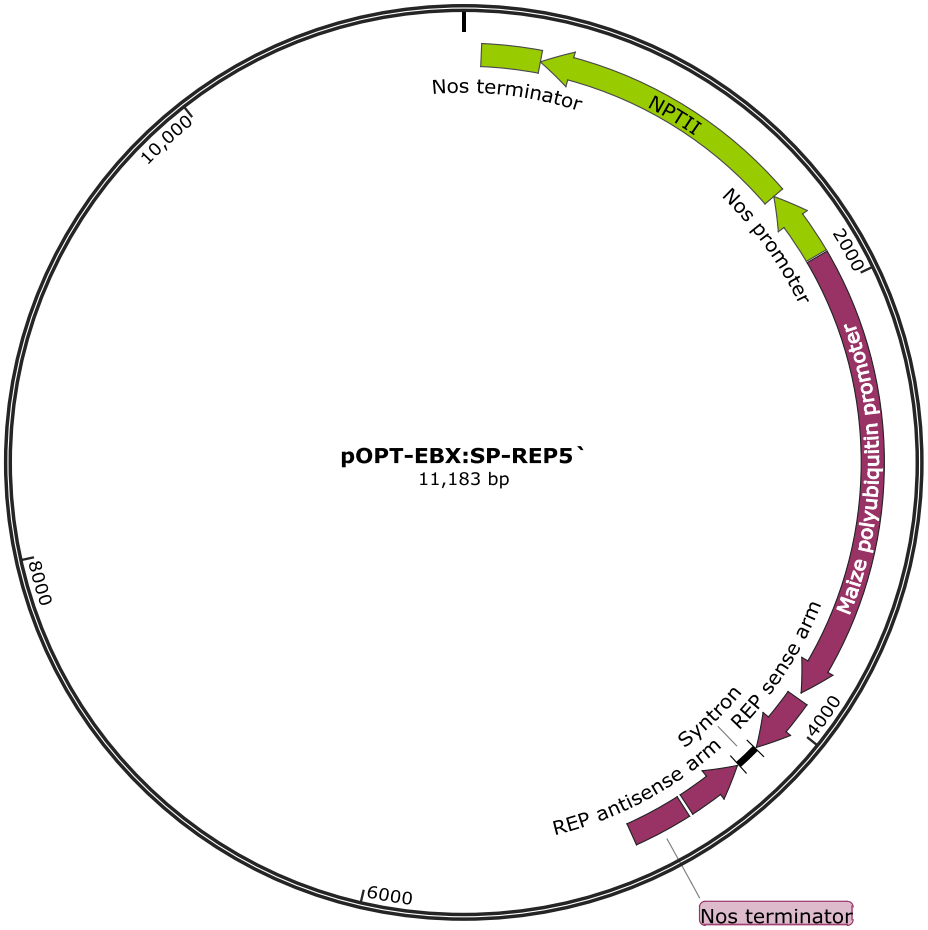


Figure 3.2: Map of a modified pOPT-EBX binary vector using BBTV SP Rep5' as an example. Expression of the hairpin cassette (maroon) is under the control of the Ubi promoter and nosT, with sense and antisense arms separated by the syntron sequence. The NPTII cassette for selection of transgenic plants is shaded green.

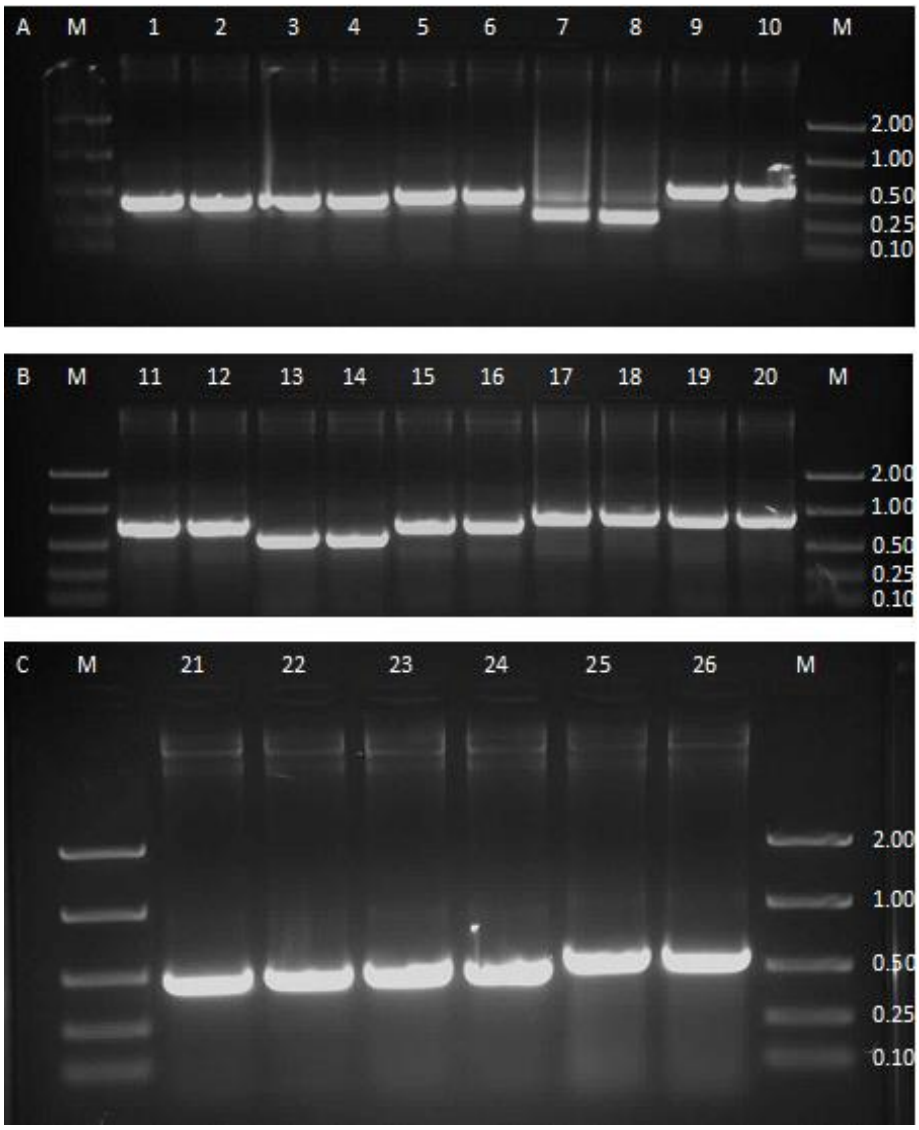


Figure 3.3: PCR testing for the presence of the hairpin cassettes after cloning into the binary vector. Syntron primers (reverse and forward) were used in combination with the forward primer of each hairpin fragment to amplify firstly the sense and secondly the antisense arm of each construct. The sizes of the expected amplicons, which include the syntron and one arm of each hairpin cassette, are included in the respective panel legend. A) SP-Rep5` (Lane 1 & 2; 381 bp), SP-RepIO (Lane 3 & 4; 382 bp), SP-MP (Lane 5 & 6; 442 bp), SP-NSP (Lane 7 & 8; 253 bp), SP-Rep5'NSP (Lane 9 & 10; 533 bp); B) SP-Rep5'MP (Lane 11 & 12; 722 bp), SP-RepIONSP (13 & 14; 531 bp), SP-RepIOMP (Lane 15 & 16; 593 bp), SP-Rep5'NSPMP (Lane 17 & 18; 873 bp), SP-RepIONSPMP (Lane 19 & 20; 872 bp); C) VN-Rep (Lane 1 & 2; 451 bp), VN-MP (Lane 3 & 4; 456 bp) and VN-NSP (Lane 5 & 6; 542 bp). M is the molecular marker EasyLadder 1 (Bioline) – sizes in kbp.

to investigate the efficiency of RNAi against single or multiple gene targets. The three vectors targeting the Asian subgroup were designed to target individual genes of the BBTV genome. The resulting binary vectors were then used for *Agrobacterium*-mediated transformation of banana ECS.

3.3.4 Transformation, regeneration and characterisation of transgenic bananas with hairpin constructs

The binary vectors were Agro-transformed into banana ECS. A total of five separate transformation experiments were carried out, each with varying numbers of constructs depending on ECS availability. In all experiments, some non-transformed cells were cultured (without antibiotic selection) as wild-type negative controls while some non-transformed cells were also cultured on media containing antibiotic selection. Further, as a positive control to assess transformation efficiency, ECS were also transformed with control construct, pART-Test7, which contains the GFP reporter gene driven by the constitutive CaMV35S promoter. Three days after transformation, ECS transformed with pART-Test7 were assessed for transient GFP expression using a fluorescence microscope. Regardless of the outcome of the GFP expression from the control, ECS transformed with pART-Test7 and the hairpin constructs were washed and maintained on selection media to allow embryo formation, germination and plantlet regeneration.

In the first experiment, ECS derived from Dwarf Cavendish (DC) banana cell line DC35-8 were transformed using the 13 hairpin constructs as well as the control pART-Test7 construct expressing GFP. Three days post-transformation, the ECS transformed with the GFP control were assessed under fluorescence microscope, but only a few isolated green fluorescing cells were observed (Fig 3.4A) suggesting a low transformation efficiency. Nonetheless, the ECS transformed with pART-Test7 as well as those transformed with the 13 hairpin constructs were placed on media containing kanamycin and routinely subcultured to fresh and appropriate media for each specific stage of cell growth and development. After three months of growth on selection media, most of the ECS were black and had died. However, for several constructs, including pART-Test7 (3.4C), a small number of germinating embryos were observed. These were maintained on M3, M4 and M5 media for three months each, resulting in plantlet formation at about 12 months post-transformation.

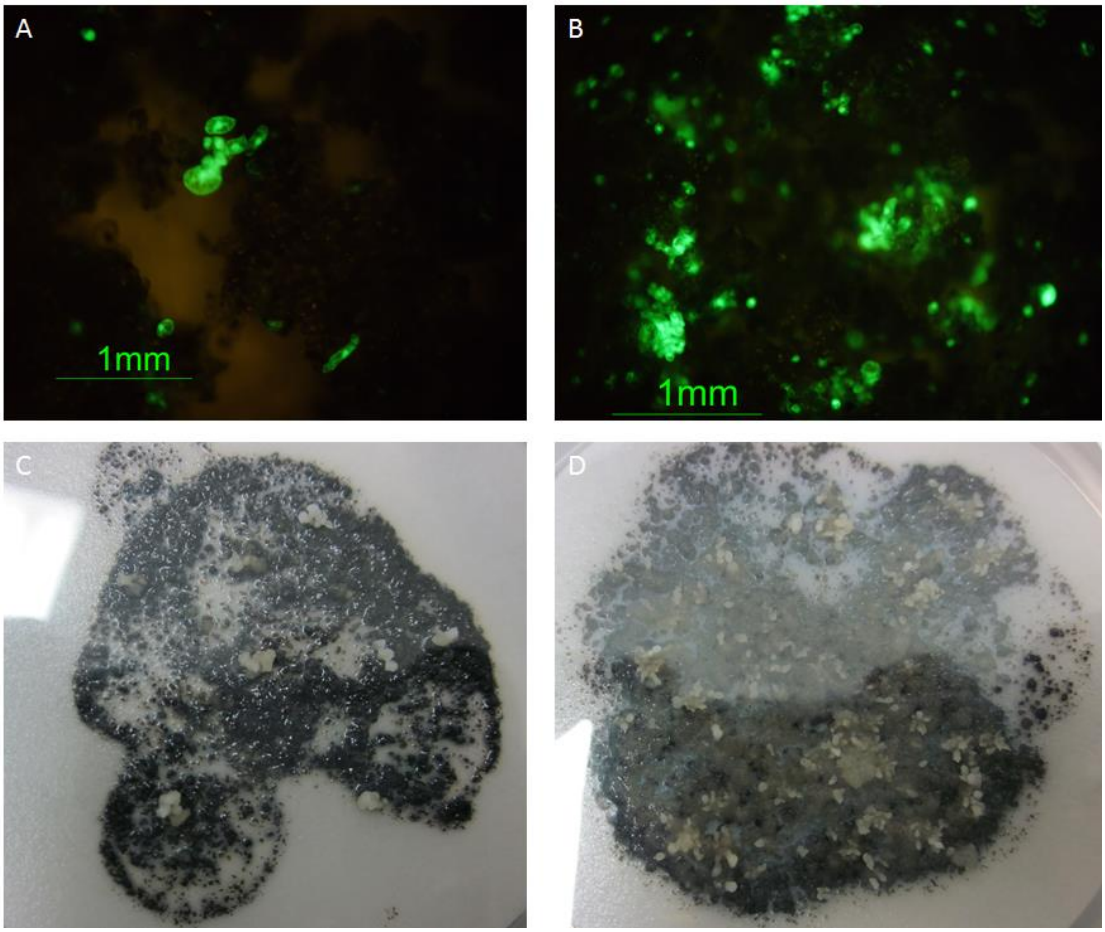


Figure 3.4: Banana ECS embryogenesis and visual detection of GFP fluorescence. A and B depict GFP fluorescence in ECS transformed with control construct, pART-Test7 from two separate transformation experiments. C and D show the germination of pART-Test7-transformed ECS growing on media from two separate transformation experiments. A significantly higher number of GFP fluorescing cells were observed and significantly more ECS germinated in the second transformation experiment (B and D, respectively).

Eventually, a total of 16 transgenic plant lines were generated from ECS transformed with the 13 binary constructs in the first experiment (Table 3.3). As expected, untransformed control cells placed on selection media progressively turned black and failed to develop into embryos, while the untransformed control cells placed on kanamycin-free media grew and developed into embryos.

In the second experiment, ECS derived from a different Dwarf Cavendish banana cell line (DC6-5) was used. This transformation experiment included seven of the binary vectors as well as the GFP control and non-transformed ECS as negative controls. Three days after transformation, transient GFP expression was again examined and, unlike the first transformation experiment, a large number of embryos were observed to be fluorescing green (Fig 3.4B). In contrast to the first transformation experiment, larger numbers of germinating embryos developed from ECS transformed with four of the hairpin vectors, as well from ECS transformed with pART-Test7 (Fig 3.4D), while for the remaining three constructs fewer regenerating embryos developed. A total of 248 transgenic plant lines were eventually generated from this transformation experiment (Table 3.3).

In the third and fourth transformation experiments, ECS of banana cv. Grand Nain cell line GN263-8 were transformed with the 13 binary constructs. Unfortunately, no plantlets regenerated from either experiment. Further, no embryos developed from the ECS transformed with the GFP control construct and all non-transformed cells (on media with or without kanamycin) also died. A fifth transformation experiment was carried out in which the SP-MP, SP-Rep5'NSPMP, SP-RepIONSPMP constructs, and a control construct containing Ubi and nosT with no hairpin cassette, were transformed into cv. Grand Naine ECS cell line GN76. In this transformation, ECS transformed with the GFP control were seen to fluoresce green when examined three days post transformation whereas untransformed ECS maintained on media with kanamycin progressively died as expected. The ECS from experiment 5 are currently growing on kanamycin selection BL media.

Table 3.3: PCR screening of putative transgenic lines regenerated from transformation experiments 1 and 2

Transformation experiment no.	Cell line	Construct	No. of plantlets regenerated	No. of plantlets selected for PCR testing	No. of PCR +ve plantlets
1	DC35-8	SP-Rep5’	1	1	1
		SP-RepIO	1	1	1
		SP-MP	2	2	2
		SP-NSP	1	1	1
		SP-Rep5’MP	1	1	1
		SP-RepIOMP	1	1	1
		SP-Rep5’NSP	2	2	2
		SP-RepIONSP	0	0	0
		SP-Rep5’NSPMP	0	0	0
		SP-RepIONSPMP	0	0	0
		VN-Rep	6	6	6
		VN-MP	1	1	1
		VN-NSP	0	0	0
		Subtotal	16	16	16
2	DC6-5	SP-Rep5’	3	3	3
		SP-MP	60	16	16
		SP-NSP	60	15	15
		SP-Rep5’MP	61	16	16
		SP-Rep5’NSP	60	15	13
		SP-RepIOMP	1	1	1
		SP-RepIONSP	3	3	3
		Subtotal	248	69	67
		Grand Total	**264**	**85**	**83**

Note: Two cell lines were used in separate transformations leading to the generation of different putatively transformed transgenic lines which were characterised for transgene presence by PCR.

3.3.5: Characterisation of regenerated plantlets

To confirm the presence of the binary construct in regenerated plantlets, genomic DNA was extracted from leaf tissue and used as template in a PCR. Hairpin-specific forward primers were used in combination with both syntron forward or reverse primers to amplify the two arms of each hairpin cassette. In addition, to eliminate the possibility of false positives due to the presence of residual *Agrobacterium,* all plantlets testing positive for the target binary construct were subsequently screened for the presence of residual *Agrobacterium* by PCR using *virC*-specific primers.

All 16 plants from transformation experiment 1 and a representative 69 plants of the 248 plants generated in transformation experiment 2 were tested by PCR for the presence of the respective binary construct. Amplicons of the expected size were observed in all plants of the 15 SP-NSP plants from experiment 2 (Fig 3.5, Table 3.4). Further, no amplicons were generated from any of the plants using *virC* primers thus excluding the presence of residual *Agrobacterium* with exception of two lines from SP-Rep5'MP which were replaced with new lines. The new lines also screened and confirmed to be free of *Agrobacterium* contamination (Appendix 2).

To determine the transgene copy number and characterise the transgene integration pattern, 78 of the 83 lines that tested positive for the respective hairpin construct were subjected to Southern blotting (Table 3.4). The remaining five lines were not tested due to insufficient tissue available at the time of sampling. Total gDNA was extracted from the transgenic plants, digested with *Hind*III (which cuts the T-DNA at a single site) and electrophoresed through agarose gels and blotted onto nylon membranes. The membranes were then hybridised with a DIG-labelled PCR probe complementary to the *npt*II gene sequence in the kanamycin selection cassette (common to all constructs).

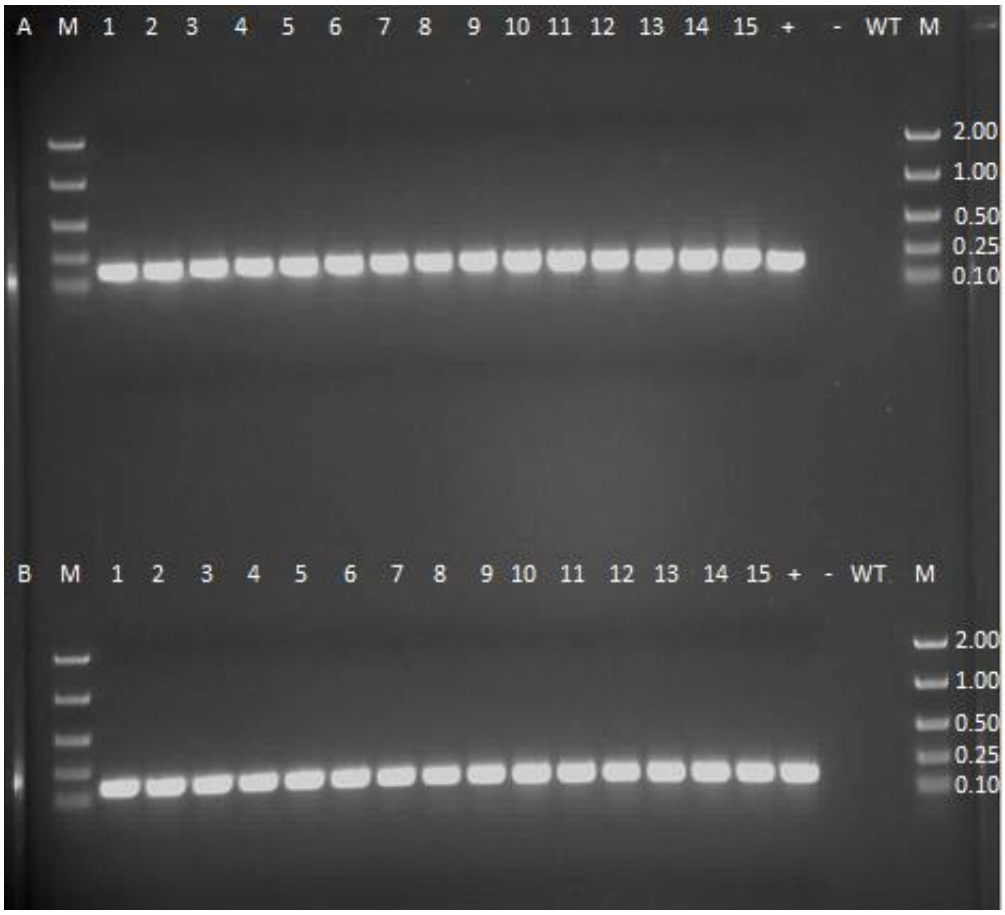


Figure 3.5: PCR analysis of SP-NSP putative transgenic lines from transformation experiment 2. A) Sense arm PCR products from lines SP-NSP1-15; and B) Antisense arm PCR products from lines SP-NSP1-15. '-' is no template control, 'WT' is wild type control and '+' is the plasmid DNA positive control. Expected size of the PCR product is 151 bp. M is the molecular marker EasyLadder 1 (Bioline) – sizes in kbp.

Table 3.4: Summary of Southern analysis of transgenic lines

Transformation Experiment No.	Cell line	Construct	No. of PCR +ve lines	Transgene copy number	No. of independent lines
1	DC35-8	SP-Rep5'	1	5	1
		SP-RepIO	1	2	1
		SP-MP	2	1	1
		SP-NSP	1	4	1
		SP-Rep5'MP	1	4	1
		SP-RepIOMP	1	1	1
		SP-Rep5'NSP	2	3,5	2
		VN-Rep	6	2-6	4
		VN-MP	1	nd[a]	-
		Total	16	-	12
2	DC6-5	SP-Rep5'	3	3-5	2
		SP-MP	16	1-6	14
		SP-NSP	15	1-5	14
		SP-Rep5'MP	16	1-5	14[b]
		SP-Rep5'NSP	13	1-5	11[c]
		SP-RepIOMP	1	nd	-
		SP-RepIONSP	3	nd	-
		Total	**67**		**55**
		Grand total	**83**		**67**

[a] 'nd' indicates lines which were not assessed by Southern blotting

[b] One line was not tested

[c] Two lines were inconclusive and were not included in further work

Of the 15 lines tested from transformation experiment 1, 12 were shown to be independent events based on their distinct hybridisation patterns (Table 3.4). The transgene copy numbers in these lines ranged from 1 to 6, with lines generated using constructs SP-MP and SP-RepIOMP shown to contain a single copy of the respective hairpin cassette (Table 3.4). The two SP-MP lines showed an identical hybridisation pattern and were thus considered to be derived from a single transformation event. Consequently, only one of the two lines was selected for subsequent experiments. The single transgenic lines containing either the SP-NSP or SP-Rep5'MP cassettes both contained four copies of the respective transgene, while the single line containing the SP-RepIO cassette had two transgene copies. Of the six VN-Rep transgenic lines tested, one contained two transgene copies while, of the remaining five lines, two had three copies and three had six copies (Table 3.4).

Of 62 lines from transformation experiment 2 that were subjected to Southern analysis, 55 were found to be independent events based on their distinct hybridisation patterns (Table 3.4). These lines were again characterised by transgene copy numbers ranging from 1 to 6. Of the four (both experiments) SP-Rep5' plants analysed, two were found to have three identical copies and the remaining two had five copies with unique integration patterns. Of the 16 SP-MP plants analysed, two plants were found to have an identical hybridisation pattern with four transgene copies (Fig 3.6B (lanes 8 and 10)) and were considered to be derived from a single transformation event. The remaining 14 plants showed a unique transgene integration pattern (Fig 3.6) and were considered to have been generated from distinct transformation events. Of these 14 plants, one line contained one copy, three lines had two copies, three lines had three copies, two lines had four copies and the remaining five lines contained six copies of the SP-MP cassette.

Of the 15 lines transformed with the SP-NSP construct, four lines contained one copy, six lines had two copies, three lines had four copies and two lines had five copies of the transgene cassette. Of the three lines with four copies, two exhibited an identical transgene integration pattern and were considered to be derived from a single transformation event. Therefore, 14 SP-NSP lines were confirmed as independent lines.

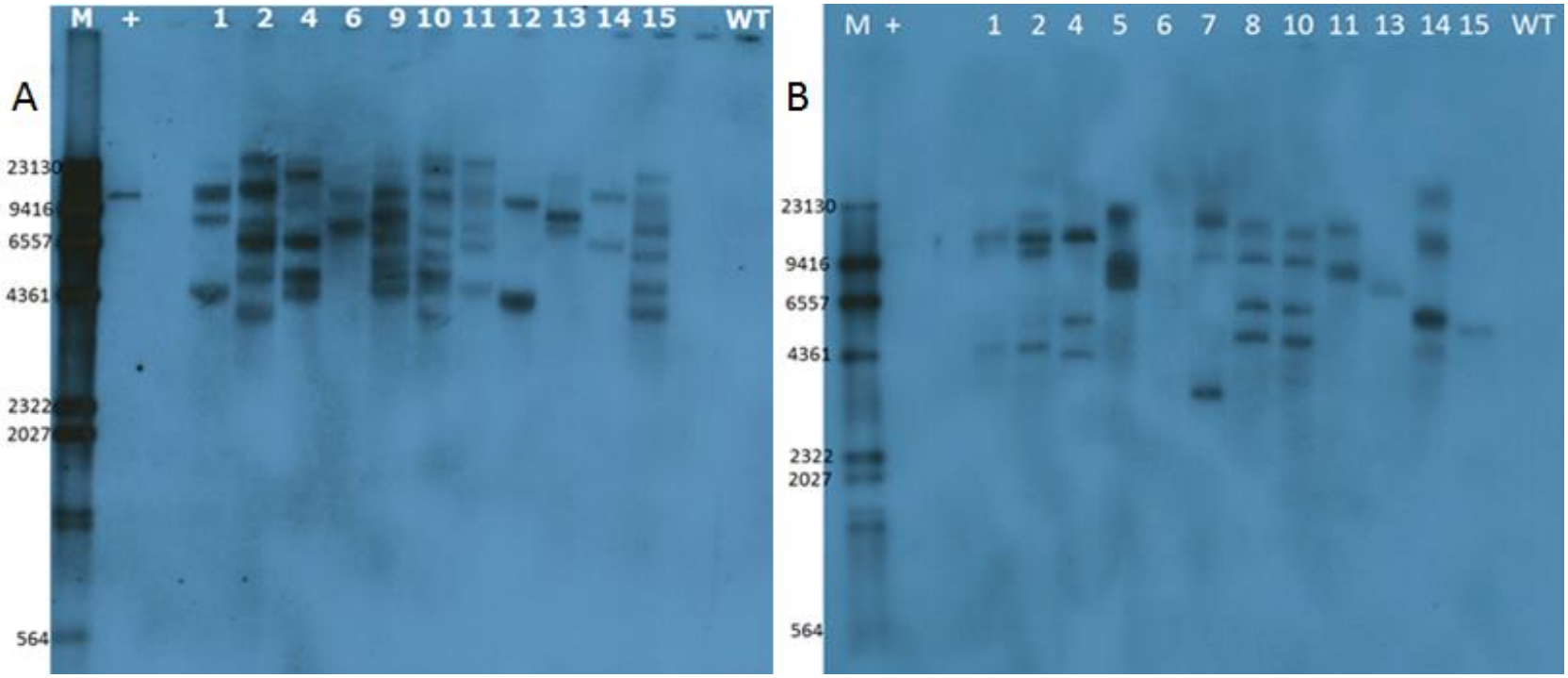


Figure 3.6: Representative results of Southern blotting of transgenic banana lines transformed with SP-MP and SP-Rep5'MP RNAi constructs using the DIG labelled NPTII probe. 'M' - is the DIG-labelled molecular marker, '+' is the positive control (50 ng of plasmid DNA), 'WT' is non-transgenic control; A) lanes 1-11 represent SP-MP transgenic lines (note – not all lines from this construct are shown in the figure); B) lanes labelled 1-15 represent SP-Rep5'MP transgenic lines. Independent transgenic lines are characterised by differing hybridisation signal patterns and/or a varying number of transgene copies. In Figure B lanes 8 and 10 are not independent as they have the same hybridisation signal patterns.

Analysis of the 16 lines transformed with the SP-Rep5'MP construct revealed that three lines contained one copy, four lines had two copies, two lines had three copies, five lines had four copies and one line had five copies of the transgene. All tested lines exhibited distinct integration patterns except for two of the lines with four copies which exhibited an identical integration pattern and were not considered independent events (Fig 3.6). Therefore, fourteen independent lines containing the SP-Rep5`MP construct were identified. Finally, of the 13 lines transformed with the SP-Rep5'NSP analysed, six lines harboured one copy of the transgene, five lines had two copies while the results from the analysis of the remaining two lines were inconclusive and they were not included in further work (Appendix 2).

3.3.6 RT-PCR analysis to detect gene transcription from hairpin cassettes in transgenic plants

To detect the presence of hairpin-specific transcripts from selected lines, total RNA was extracted from leaf samples and treated with DNase prior to analysis by RT-PCR using primers specific for each respective hairpin cassette. Due to the small size of some plants, only the single SP-MP line from transformation experiment 1 (Table 3.3) and 22 independent lines from transformation experiment 2 (10 lines from SP-MP and 12 lines from SP-Rep5`MP) were analysed. When total RNA extracts from wild-type banana plants were tested by RT-PCR using construct-specific primers, no amplicons were observed as expected. However, a strong product of the expected size of 440 bp was amplified from the SP-MP line from transformation experiment 1, while products of the expected sizes of 440 bp and 720 bp were amplified in all of the 10 and 12 SP-MP and Rep5'MP transgenic lines tested, respectively (Fig 3.7; Appendix 2).

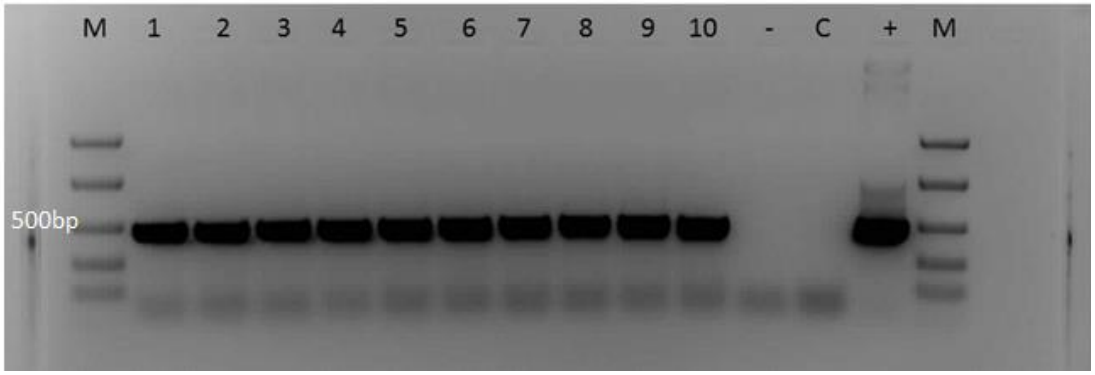


Figure 3.7: RT-PCR analysis of 10 SP-MP transgenic banana lines from transformation experiment 2. The primers used amplify a 440 bp region of the hairpin arm and syntron. Lane '-' no template control, '+' positive control-plasmid DNA harbouring the SP-MP construct, 'C' wild-type control and 'M' is EasyLadder 1 (Bioline).

3.4 Discussion

BBTV is one of the most devastating pathogens of banana crops worldwide and there are limited reports of resistance to BBTV in cultivated bananas. Therefore, there is an urgent need to generate bananas with resistance to BBTV. In an attempt to address this problem, a suite of binary constructs containing hairpin cassettes targeting the genes of three distinct BBTV DNAs was constructed to assess the effect of using different virus sequences to generate RNAi-based resistance. In addition, several multimeric constructs were prepared to determine if targeting more than one virus gene sequence provided enhanced resistance. Finally, several constructs were prepared using sequences derived from an Asian subgroup of BBTV isolates to determine if a broad spectrum resistance could be generated.

In the first and second transformation experiments, binary vectors were Agro-transformed (Khanna *et al.*, 2004) into Dwarf Cavendish banana ECS. Following selection and regeneration, 264 putative transgenic lines were generated from which 85 were selected for molecular characterisation. When tested by PCR, 83 of the lines were found to contain the respective hairpin cassette, confirming a high efficiency of transformation. To increase the available lines for some constructs, three additional transformation experiments were carried out. In the third and fourth experiments, no plants were regenerated from either transformed or control ECS, probably as a result of the low viability of these cell lines. The fifth transformation experiment also used ECS from cv. Grand Nain and a considerable number of plantlets are currently regenerating. Due to time constraints, however, these plants were unable to be characterised during this study.

Southern blotting was carried out to confirm that PCR positive transgenic lines represented independent transformation events. This analysis identified a diverse collection of T-DNA integration patterns with transgene copy numbers ranging from one to six. Fifteen of the lines characterised were shown to contain a single copy of the respective hairpin cassette and the observation of distinct integration patterns in most cases confirmed 67 independent lines had been generated (Fig 3.6 and Table 3.2). These findings are consistent with previous studies where between one and five transgene copies were reported in transformation of banana ECS (Khanna *et al.*, 2004; Pérez-Hernández *et al.*, 2006). Since not all the plant lines generated were tested for transgene integration, additional independently transformed

lines may be available for analysis and virus challenge in the future. It is also highly likely that more lines will be generated from the fifth transformation experiment.

To confirm the expression of the hairpin cassettes, selected lines were tested by RT-PCR. Independent lines transformed with SP-MP and SP-Rep5'MP constructs were screened and analysis confirmed the expression of the cassettes in all transgenic lines tested. Due to time constraints the transgenic lines generated using the other constructs presented in Table 3.3 were not tested by RT-PCR, therefore, these transgenic lines will need to be screened to confirm expression of their respective hairpins in the future.

Three BBTV genes were targeted for silencing in this study, with the major focus on the BBTV-R-encoded Rep gene. Recent studies have reported the generation of BBTV resistant transgenic lines using a variety of different strategies targeting the viral Rep gene (mutated Rep sequence, antisense strand of Rep, full length Rep, inverted repeat of Rep sequence and hpRNAs) (Borth *et al.*, 2011; Shekhawat *et al.*, 2012). In the study by Borth *et al.* (2011), transgenic virus resistance was generated using mutant, antisense, partial and full Rep gene constructs against a Hawaiian BBTV isolate. Shekhawat *et al.* (2012) generated transgenic lines expressing siRNAs against an Indian BBTV isolate using hairpin constructs targeting the Rep gene. The design of constructs made by Shekhawat *et al.* (2012) involved the use of full length Rep complementary sequences separated by a spliceable intron to form a hairpin RNA transcript capable of inducing RNA silencing of the viral Rep mRNA. The strategy used in the present study is unique in that conserved sequences were used to develop less-than-full length hpRNA vectors targeting both individual and multiple genes for broad spectrum resistance against South Pacific and Asian group of BBTV isolates. In addition, unlike the design employed in the study by Shekhawat *et al.* (2012), the current study utilised constructs based on partial Rep gene sequences and with start codons deleted to eliminate the possibility of viral protein formation. However, it is worth noting that in the study by Shekhawat *et al.* (2012), the antisense arm of the intron hairpin RNA constructs was located downstream of the maize polyubiquitin promotor to stop translation of the small internal open reading frame. The constructs prepared in the current study also comprised a synthetic intron separating the sense and antisense arms to enhance formation of a hairpin

transcript and improve the effectiveness of the silencing mechanism (Shekhawat *et al.*, 2012; Wesley *et al.*, 2001).

The second BBTV gene targeted for silencing in the current study was DNA-M. In addition to its role in viral DNA movement within infected cells, the DNA-M encoded MP also acts as a suppressor (Amin et al., 2011; Niu et al., 2009) of post transcriptional gene silencing (PTGS) (Incarbone & Dunoyer, 2013; Pumplin & Voinnet, 2013; Shimura *et al.*, 2013; Várallyay & Havelda, 2013). Targeting these viral suppressors of PTGS for silencing themselves may improve the effectiveness of RNA-mediated virus resistance.

The gene from BBTV DNA-N was also targeted for silencing in the current study. DNA-N is one of the most conserved BBTV genomic components and its gene product plays an important role in the transport of viral DNA between the nucleus and cytoplasm. This DNA component has not previously been assessed as a target for resistance. By down-regulating DNA-N gene expression and, therefore, BBTV intracellular transport, it was hypothesized that this would significantly impair the life cycle of the virus.

Thus, the current study has generated a large number of transgenic banana lines, expressing a diverse range of hairpins targeting different genes of BBTV that may be useful in efforts towards development of BBTV resistance in banana. These lines now require small-scale glasshouse testing to determine their resistance or susceptibility to BBTV infection to select promising elite lines for later field trials.

CHAPTER 4

GLASSHOUSE ASSESSMENT OF TRANSGENIC BANANAS TRANSFORMED WITH HAIRPIN CONSTRUCTS TARGETING BBTV

4.1 Introduction

In the previous chapter, 67 independent transgenic banana plants expressing hairpin cassettes targeting a range of different BBTV target sequences were generated following *Agrobacterium*-mediated transformation. Previous studies investigating the use of hairpin cassettes to generate virus resistance have shown that abiotic conditions, differences in transgene integration sites and levels of hairpin cassette expression can affect the ability of the construct to confer resistance (Di Nicola *et al.*, 2014; Zhang *et al.*, 2013; Zhang *et al.*, 2011). For example, in the study conducted by Zhang *et al.* (2013), *Maize dwarf mosaic virus* (MDMV) resistance was generated by expression of hairpin constructs targeting the MDMV protease P1 gene in maize plants. They showed that of 12 transgenic lines containing one copy of the transgene, 50% were resistant to infection while the other 50% showed "intermediate" resistance. Further, all transgenic lines containing more than one transgene copy showed either "intermediate" resistance or were susceptible. As such, it is necessary to test as many independently transformed plant lines as possible to increase the likelihood of generating plant lines with resistance to the target virus.

Preliminary testing of transgenic plants for virus resistance is often done under confined conditions to identify the best candidates for subsequent field trials (Shekhawat *et al.*, 2012; Yang *et al.*, 2014; Zhang *et al.*, 2013). Glasshouse trials allow for rapid screening and compliance with regulatory limitations on the limited release of genetically modified organisms to the environment. Since BBTV is a quarantine pest and is under active control in Queensland, screening of the transgenic lines for their response to virus infection is more easily carried out under glasshouse conditions. Glasshouse confinement is also preferable as it prevents the escape of viruliferous aphids which vector BBTV and may disseminate the virus to banana plants in surrounding areas. Several transgenic lines were generated in the previous chapter using DNA sequences derived from a Vietnamese BBTV isolate. Due to quarantine regulations, screening for BBTV resistance in these lines can only be done using an Australian BBTV isolate. This screening will provide direct evidence for the future

selection of BBTV sequences that can be used to generate broad spectrum resistance against both South Pacific (SP) and Asian subgroup isolates.

To assess plants for resistance, both visual inspections for symptoms as well as molecular testing can be used. Although bunchy top symptoms are very characteristic, testing of plants using a sensitive diagnostic technique, such as polymerase chain reaction (PCR) is still necessary. Further, information on the level of plant resistance/tolerance may also be obtained using a quantitative measure of the levels of virus DNA in infected plants. Real-time PCR (rtPCR) is now widely used to quantify the level of virus replication in host plants, with the level of resistance determined by comparison of virus levels in wild-type versus transgenic plants (Momonoi *et al.*, 2015; Wang *et al.*, 2014). Ideally, however, resistance would result in complete immunity from virus infection. Approaches based on RNAi to generate virus resistance can also be characterised based on the expression of transgene-associated, small-interfering RNAs (siRNAs), (Vanderschuren *et al.*, 2007; Wang *et al.*, 2014; Xia *et al.*, 2014) these siRNAs being hallmarks for activation of the RNAi pathway (Shekhawat *et al.*, 2012; Zhang *et al.*, 2011).

Although transgenic banana lines have been generated in this study, their reaction to inoculation with BBTV needs to be evaluated. Therefore, the aim of this chapter was to multiply the transgenic banana lines for evaluation in glasshouse inoculation trials.

The specific objectives of this chapter were to:

1. Evaluate the resistance of transgenic banana lines against BBTV infection.
2. Determine the levels of virus DNA accumulation in inoculated transgenic banana lines.
3. Confirm the presence of transgene-specific small RNAs in resistant transgenic banana lines.

4.2 Materials and methods

4.2.1 Tissue culture multiplication

Transgenic and wild-type Dwarf Cavendish bananas were cultured on multiplication media described in Appendix 1. Every four weeks the plantlets were transferred to fresh multiplication media until at least 15 replicates were obtained, a process generally taking 12-16 weeks. Following multiplication, plantlets were transferred to rooting media (section 3.2.5) for an additional 8-12 weeks to encourage root development.

4.2.2 Glasshouse acclimatisation

Tissue-cultured plantlets were removed from flasks, washed free of culture media and planted in seedling tubes (5 cm plastic pots) containing Yates Premium Potting Mix. Plants were placed into plastic trays and watered, then sealed under plastic film for two weeks to maintain high humidity and so reduce transplanting stress. The plastic film was progressively opened during weeks three to four, after which the plastic film was removed completely and plantlets introduced to environmental conditions within the glasshouse. After the sixth week plants were transferred to 15 cm pots containing Premium Potting Mix supplemented with "Osmocote Plus'' slow release complete nutrient fertilizer and allowed to grow for a further four to five weeks.

4.2.3 BBTV inoculation

A local south-east Queensland BBTV isolate was used to inoculate banana plants. Black banana aphids (*P. nigronervosa*) were maintained on Dwarf Cavendish plants under natural glasshouse conditions at ambient temperature/humidity. BBTV-infected bananas were maintained by inoculation of 12 week-old banana plants at two to three month intervals.

Aphids reared on BBTV-infected plants were used directly for inoculation of trial plants. Approximately 20 aphids were transferred to each test plant by removing leaf or petiole tissue from a BBTV-infected plant on which aphids were feeding and transferring this tissue onto the test plant. Instinctive natural migration by aphids thereby ensured no physical damage to the aphid's stylets. After an inoculation period of two weeks, plants were sprayed with pyrethrum insecticide. Following inoculation, transgenic plants and non-transgenic controls were maintained under natural glasshouse conditions. In all trials, unless

otherwise indicated, 12 test plants were inoculated using viruliferous aphids and three plants were maintained as non-inoculated controls.

4.2.4 Monitoring for symptom development

Plants were monitored for symptom development over a period of 12 weeks. At the completion of this period, all plants were examined for the characteristic symptoms of bunchy top and symptoms recorded. Leaf tissue from the youngest unfurled leaf was collected from all plants at week 12 to test for the presence of BBTV using PCR.

4.2.5 PCR detection of BBTV

Total nucleic acid extracts were prepared (section 2.5.1) and diluted to a concentration of 50 ng/µL. PCR was then performed as described in section 2.4.2 using primers which detect DNA-C (DNA-C-fwd 5` GTTCTCTTGGAGTGATTGTCAG 3` and DNA-C-rev 5` CTTAATCTCTCTCTT GACATCG 3`) and amplicons visualised by electrophoresis through 1.5% agarose gels stained with 0.5x SYBR safe DNA gel stain. Leaf tissue samples from non-inoculated control plants and virus-infected banana plants described in 4.2.3 above were used as negative and positive controls, respectively.

4.2.6 Absolute quantification by real-time (rt)PCR

rtPCR was carried out as described in section 2.4.3 using primers specific for BBTV DNA-S (DNA-S-fwd 5` TATGGCAGCAAGGCGGCAAC 3` and DNA-S-rev 5` TCCGGGCTTCACCTTGCACA 3`). The 105 nt rtPCR target amplicon was cloned into pGemT-Easy and purified plasmid DNA was then used to prepare a standard curve for absolute quantification as described by Xue *et al.* (2014). During the course of the rtPCR optimisations, it was noted that non-specific amplification (background) occurred even in control plants after 35 cycles (the lowest limit of detection based on plasmid standard). Consequently to eliminate the effect of background amplification, all measurements were corrected by subtracting the average copy number calculated in wild-type non-inoculated plants from the average of all inoculated plants. The virus DNA levels were subjected to statistical analysis using ANOVA to identify results which were significantly different. For each line screened using rtPCR, three individual plants were sampled and each sample was tested in triplicate. Leaf tissue samples from wild-type virus-free Dwarf Cavendish plants were used as negative controls and

samples from wild-type BBTV-infected Dwarf Cavendish plants were used as positive controls.

4.2.7 Northern blotting to detect small RNAs

Northern blotting was carried out as described in section 2.7. Briefly, RNA was separated in a 17% polyacrylamide gel followed by electro-blotting onto positively charged nylon membranes and subsequent immobilisation by UV cross-linking. A P^{32} radioactively labeled RNA probe was synthesised from BBTV DNA-M sequence cloned into pGemT-Easy (Promega). RNA transcripts were synthesised using T7 and SP6 polymerase and a P^{32} radiolabelling kit (PerkinElmer). As a control for the detection of 21 nt siRNAs, a miRNA159-specific probe was kindly provided by Dr. Julia Bally (QUT). Following hybridisation to detect BBTV DNA-M small RNAs, the membrane was stripped and re-probed with the miRNA159-specific probe. Since miRNA159 is 21 bp, its hybridisation signal in the membrane was used to approximate the size of DNA-M-specific small RNAs co-located on the blot.

4.3 Results

4.3.1 Glasshouse experiment 1

In the first inoculation trial, 10 independently transformed transgenic lines containing seven different hairpin constructs were assessed (Table 4.1). These 10 transgenic lines were derived from transformation experiment 1 (section 3.3.4). Although more than 10 lines were originally generated (Table 3.4), only 10 were available for screening due to losses that occurred during multiplication, rooting and acclimatisation. Due to insufficient numbers of viruliferous aphids, the 10 lines were divided into two groups for each inoculation trial, with separate control plants for each group. Further, each of the 10 transgenic lines was assessed in three separate (repeat) screening trials staggered over a period of eight months. A total of 12 replicates per transgenic line and 12 wild-type controls were inoculated per trial. Non-inoculated control plants for each transgenic line were maintained in a separate glasshouse compartment. Prior to inoculation, DNA was extracted from the leaves of all plants and tested for the presence of BBTV by PCR using DNA-C specific primers. No amplicons were generated from any of the extracts.

Table 4.1: Reaction of wild-type and transgenic Dwarf Cavendish banana lines to BBTV infection in glasshouse experiment 1

Inoculation group[a]	Construct/Line	Trial 1			Trial 2		Trial 3	
		No. of plants with symptoms	No. of PCR positive plants	Disease incidence (%)	No. of plants with symptoms	Disease incidence (%)	No. of plants with symptoms	Disease incidence (%)
1	SP-Rep5'-1	11	11	92	9	75	10	83
	SP-RepIO-1	9	9	75	11	92	11	92
	SP-MP-2	0	0	0	0	0	NT[b]	NT
	SP-NSP-1	10	10	83	12	100	12	100
	SP-Rep5'NSP-1	12	12	100	12	100	12	100
	Wild type	10	10	83	12	100	12	100
2	VN-Rep-1	8	9	67	8	67	10	83
	VN-Rep-2	11	11	92	12	100	12	100
	VN-Rep-3	11	11	92	12	100	12	100
	VN-Rep-4	7	7	58	6	50	5	42
	VN-MP-1	12	8[c]	100	12	100	12	100
	Wild type	9	9	75	12	100	12	100

[a] Plant lines were inoculated in two groups, with separate wild type control plants included with each group.
[b] Line SP-MP-2 was not included in Trial 3 due to insufficient replicates.
[c] Four plants of line VN-MP-1 died after developing symptoms of bunchy top and no sample was available for PCR testing.

In all three trials, initial characteristic symptoms of bunchy top were observed five weeks post-inoculation on some inoculated, wild-type banana control plants as well as plants from several transgenic lines (Figure 4.1). These symptoms included chlorotic leaf margins, dark green streaks along the petioles, narrowing and bunching of newly formed leaves and a general stunting of the entire plant. In some instances, such as with four replicates of transgenic line VN-MP-1, infection resulted in plant death. Disease incidence was determined by recording the number of replicates with symptoms expressed as a percentage of the total number of replicates inoculated per transgenic line. In wild-type control banana plants, a disease incidence of 75-83% was observed 12 weeks post-inoculation in the first inoculation trial, whereas 100% infection of the controls was observed in trials two and three (Table 4.1). Disease incidence in the transgenic lines varied considerably. Generally, disease incidence was high, with an averaged disease incidence of 50% or greater in almost all transgenic lines over the three screening trials comprising glasshouse experiment 1 (Fig. 4.2). In some instances, such as lines SP-Rep5`NSP-1 and VN-MP-1, a 100% disease incidence was observed in all three trials. In contrast, no symptoms were observed in any plants of line SP-MP-2 (tested in trial 1 and 2, but not trial 3), and these remained symptomless for 10 months post-inoculation at which time the plants were destroyed. None of the non-inoculated control plants from both wild-type and transgenic treatments developed symptoms of bunchy top throughout all the trials (Fig. 4.3).

To test for the presence of BBTV in plants in glasshouse trial 1, DNA was extracted from leaf samples and used in a PCR with primers designed to amplify a 647 bp fragment of BBTV DNA-C. An amplicon of the expected size was observed in samples from all 10 inoculated wild-type control plants showing bunchy top symptoms, while no amplicons were generated from samples derived from the symptomless, non-inoculated transgenic or wild-type control banana plants (Fig. 4.4). The same correlation between bunchy top symptoms and BBTV detection by PCR was seen in extracts taken from all of the transgenic lines with the single exception of one replicate of line VN-Rep-1, which did not show symptoms but which tested PCR positive (Table 4.1). Due to the strong correlation between symptoms and PCR detection of BBTV in the first trial, none of the plants in the second and third inoculation trials for these lines were PCR tested.

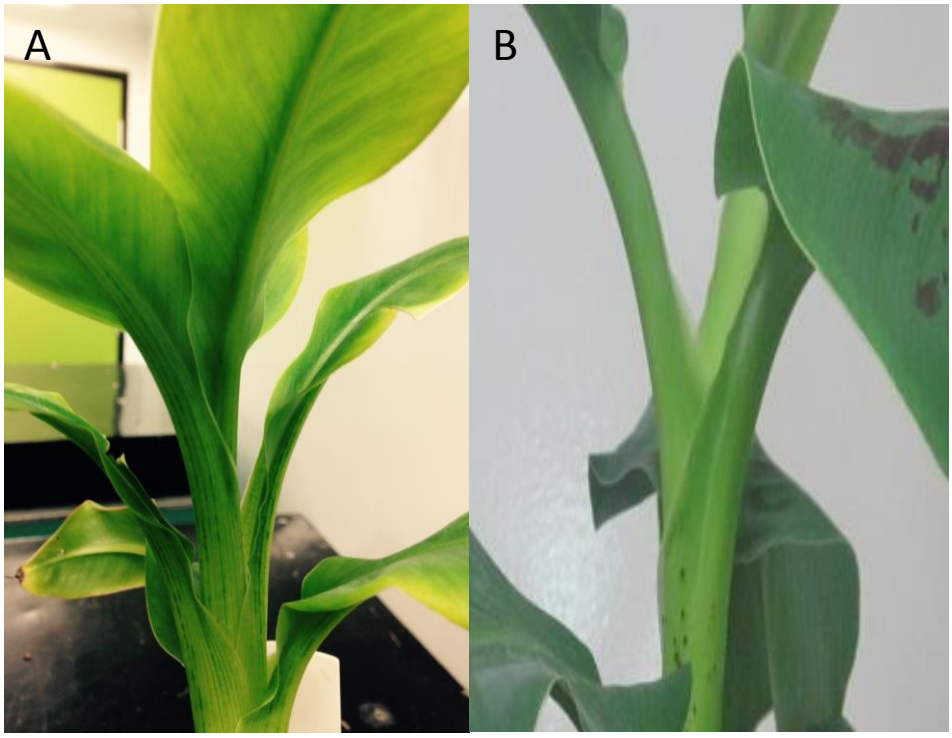


Figure 4.1: A) Characteristic symptoms of BBTV infection in banana. Dark green streaks are present on the abaxial surface of leaves and petioles and chlorotic margins have developed on the leaves; B) Healthy plant without symptoms.

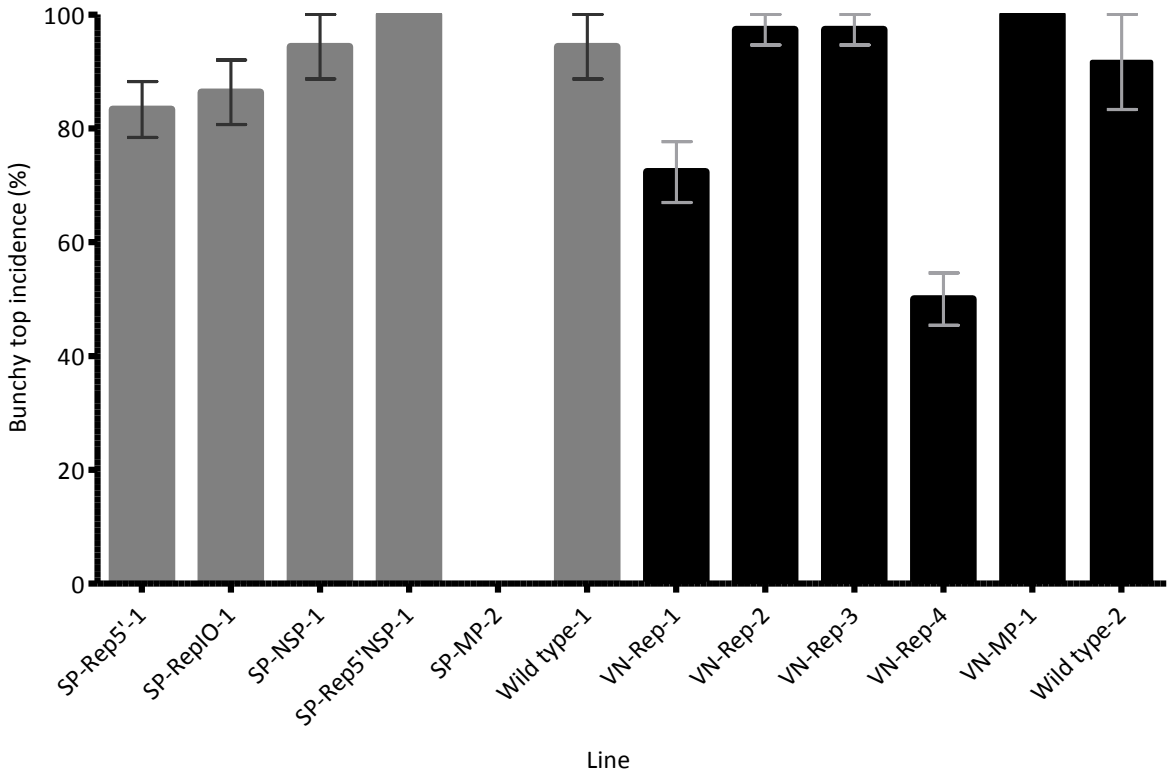


Figure 4.2: Average bunchy top incidence in glasshouse experiment 1. The error bars represent standard deviation from the mean of bunchy top incidence in 12 replicates of each transgenic line and wild type controls.

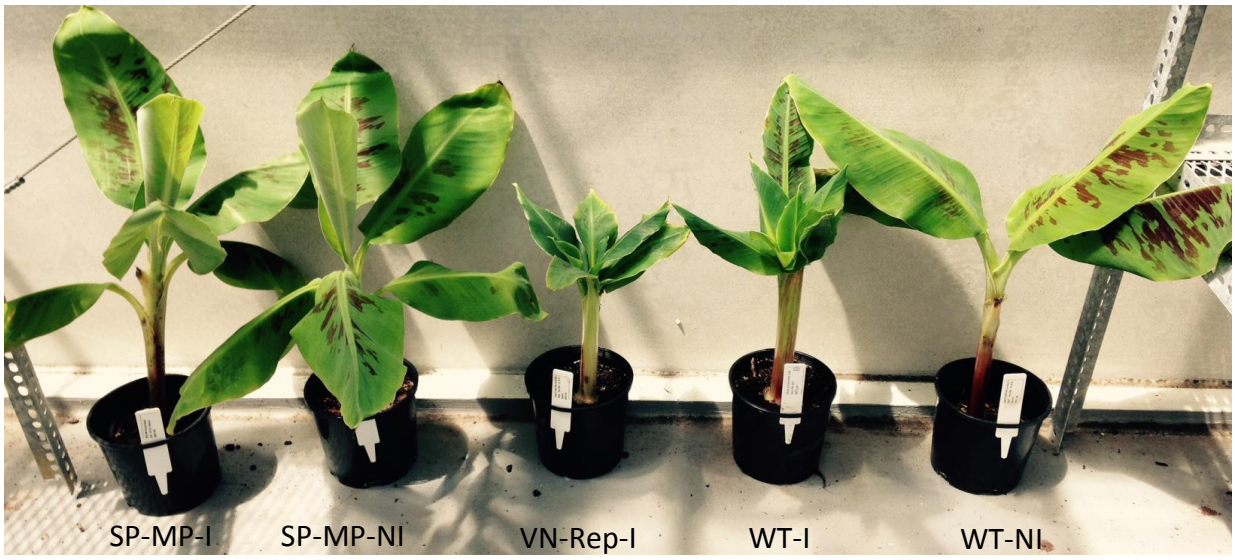


Figure 4.3: Representative photograph of plants from glasshouse experiment 1. SP-MP-I is an inoculated plant of line SP-MP-2 with no symptoms, while SP-MP-NI is the corresponding non-inoculated control; VN-Rep-I is an inoculated plant of line VN-Rep-1 with symptoms; WT-I is an inoculated wild-type control with symptoms and WT-NI is the corresponding wild-type non-inoculated control.

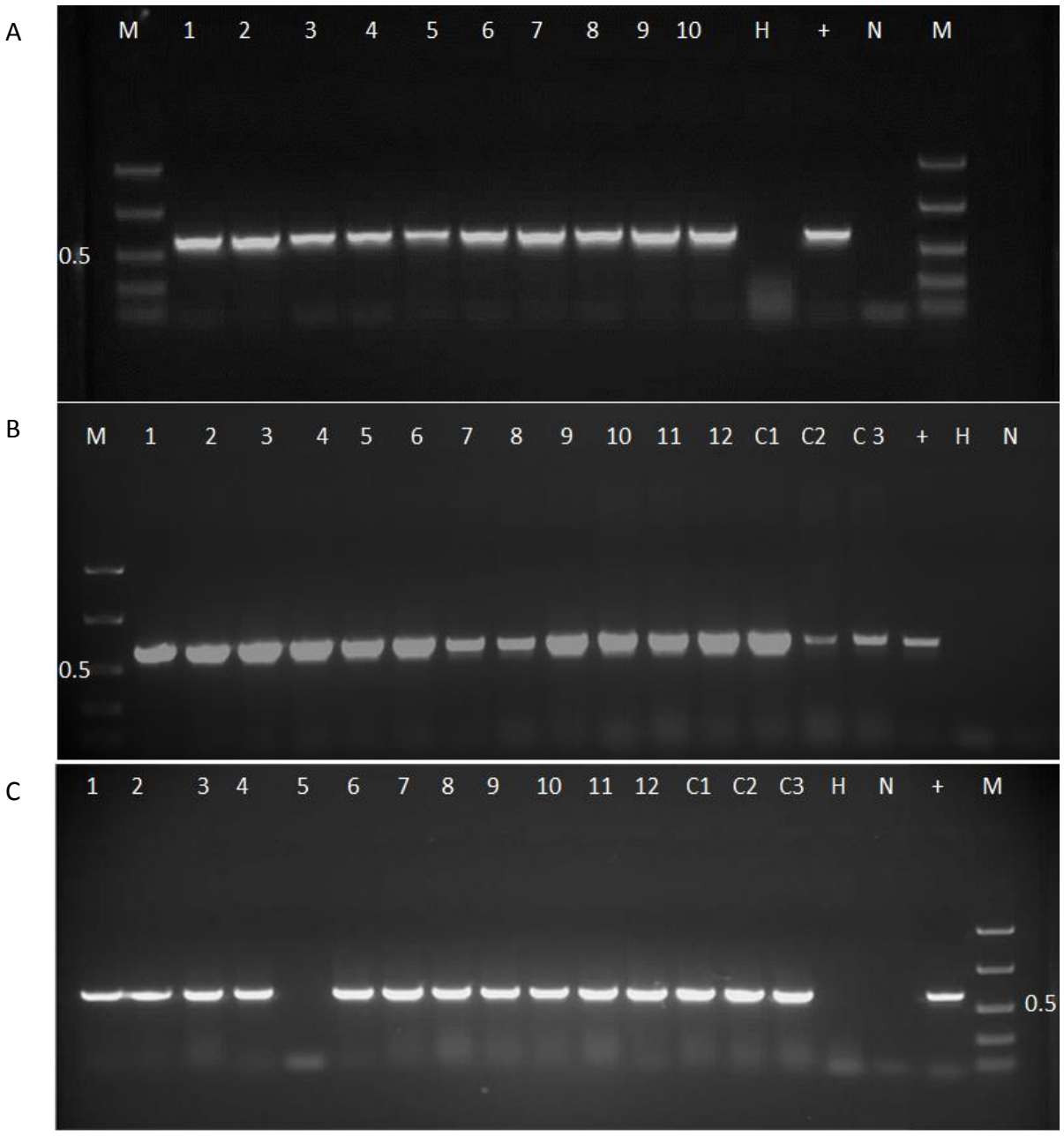


Figure 4.4: PCR detection of BBTV in banana plants in glasshouse experiment 1. A) 1-10 are inoculated wild-type control plants; B) 1-12 are replicates of line SP-Rep5'NSP-1; and C) 1-12 are replicates of line VN-Rep-2. Samples with amplicons of the expected size of 637 bp are positive for BBTV. M is EasyLadder 1 (Bioline), H is the non-inoculated control, N is the no-template control and + is the known BBTV-infected positive control. C1-C3 in panels B and C are samples from BBTV-infected wild type controls.

4.3.2 Glasshouse experiment 2

In glasshouse experiment 2, screening focussed on the SP-MP and SP-Rep5'MP transgenic lines derived from transformation experiment 2 described in Chapter 4 (Table 4.2). In addition, line SP-MP-2 which had been previously screened in trials 1 and 2 in glasshouse experiment 1 was included for a third glasshouse challenge. Due to time constraints, only a single inoculation trial for these lines was done. As with glasshouse experiment 1, the plants were divided into groups and tested in separate trials based on aphid availability. Twelve plants of each line were tested except for lines Rep5`MP-6 and Rep5`MP-13 for which only nine replicates were available (Table 4.2).

As before, initial symptoms typical of bunchy top were observed at about five weeks post-inoculation in both wild-type control plants as well as plants from several transgenic lines. Disease incidence in the inoculated wild-type plants at 12 weeks post-inoculation was 100% for two of the three groups of plants inoculated (group one and group two), however, in group three only 1/12 wild-type plants developed symptoms at 12 weeks post-inoculation (Table 4.2). In the first two inoculation groups, which included 11 SP-MP lines and eight SP-Rep5`MP lines, respectively, typical bunchy top disease symptoms developed in plants from six transgenic lines, with a final disease incidence ranging from 8 to 25%. The remaining lines in these two groups had no replicates with symptoms at 12 weeks post-inoculation. Of the four SP-Rep5`MP transgenic lines inoculated in the third group, no plants developed symptoms. When DNA extracted from leaf samples was tested for BBTV by PCR, a 100% correlation was observed between detection of BBTV and presence of symptoms, while all non-inoculated control plants remained free of symptoms and tested negative for BBTV.

Table 4.2: Reaction of wild-type and transgenic banana lines to BBTV infection in glasshouse experiment 2

Inoculation group[a]	Construct	No. of plants with symptoms	No. of PCR positive plants	Disease incidence (%)
1	SP-MP-1	0	0	0
	SP-MP-2	0	0	0
	SP-MP-4	0	0	0
	SP-MP-6	0	0	0
	SP-MP-9	0	0	0
	SP-MP-10	1	1	8
	SP-MP-11	2	2	17
	SP-MP-12	3	3	25
	SP-MP-13	0	0	0
	SP-MP-15	3	3	25
	SP-MP-2 [b]	0	0	0
	Wild type	12	12	100
2	SP-Rep5'MP-1	3	3	25
	SP-Rep5'MP-2	1	1	8
	SP-Rep5'MP-4	0	0	0
	SP-Rep5'MP-5	0	0	0
	SP-Rep5'MP-6[c]	0	0	0
	SP-Rep5'MP-7	2	2	17
	SP-Rep5'MP-14	0	0	0
	SP-Rep5'MP-19	0	0	0
	Wild type	12	12	100
3	SP-Rep5'MP-8	0	0	0
	SP-Rep5'MP-10	0	0	0
	SP-Rep5'MP-11	0	0	0
	SP-Rep5'MP-13[c]	0	0	0
	Wild type	1	1	8

[a] Plant lines were inoculated in three groups, with separate wild type control plants included with each group.

[b] Line SP-MP-2 from transformation experiment one was included for the third repeat screening (see Table 5.1 above).

[c] Only nine replicates were inoculated for these two lines.

4.3.3 Quantification of BBTV DNA in plants from glasshouse experiment 1

To determine the level of BBTV accumulation in wild-type and transgenic plants assessed in glasshouse experiment 1, DNA was extracted from selected plants and analysed by rtPCR. For each transgenic line, three replicates were randomly selected from the plants which had previously tested positive for BBTV DNA-C using conventional PCR, except for line SP-MP-2 where all replicates had tested negative for BBTV (Table 4.1). A standard curve was constructed to determine the absolute quantity of BBTV DNA-S (as a representative of all BBTV DNAs) present in virus-infected wild-type and transgenic plants. A ten-fold serial dilution of plasmid DNA containing the target amplicon from BBTV DNA-S was quantified (in triplicate) and a linear regression curve was generated by plotting the mean cycle threshold (Ct) values against the log of the copy number of the target amplicon. The standard curve yielded a linear equation of y = -3.4034x + 31.687 and a perfect coefficient of correlation (R^2=1), with an amplification efficiency of 97%.

Subsequently, rtPCR was carried out to determine the accumulation of BBTV DNA-S in lines from glasshouse experiment one (trial 1). When the wild-type and transgenic plants from inoculation group 1 were assessed, no significant difference was seen between the amount of DNA-S in BBTV-infected wild-type control plants and BBTV-infected transgenic plants, with the exception of line SP-Rep5'-1 which had significantly lower DNA-S accumulation ($P<0.001$) compared to wild-type control plants (Fig 4.5). There was no DNA-S accumulation detected in the SP-MP-2 transgenic banana plants (Fig 4.5), consistent with these plants not showing bunchy top symptoms and previously testing negative for DNA-C using conventional PCR. When the wild-type and transgenic plants from lines tested in inoculation group 2 were assessed, no significant difference was again observed between the amount of DNA-S in BBTV-infected wild-type control plants and BBTV-infected transgenic plants (Fig. 4.5). Interestingly, the average DNA-S accumulation in the wild-type plants from inoculation group 2 was half the average DNA-S accumulation in the wild-type plants from inoculation group 1.

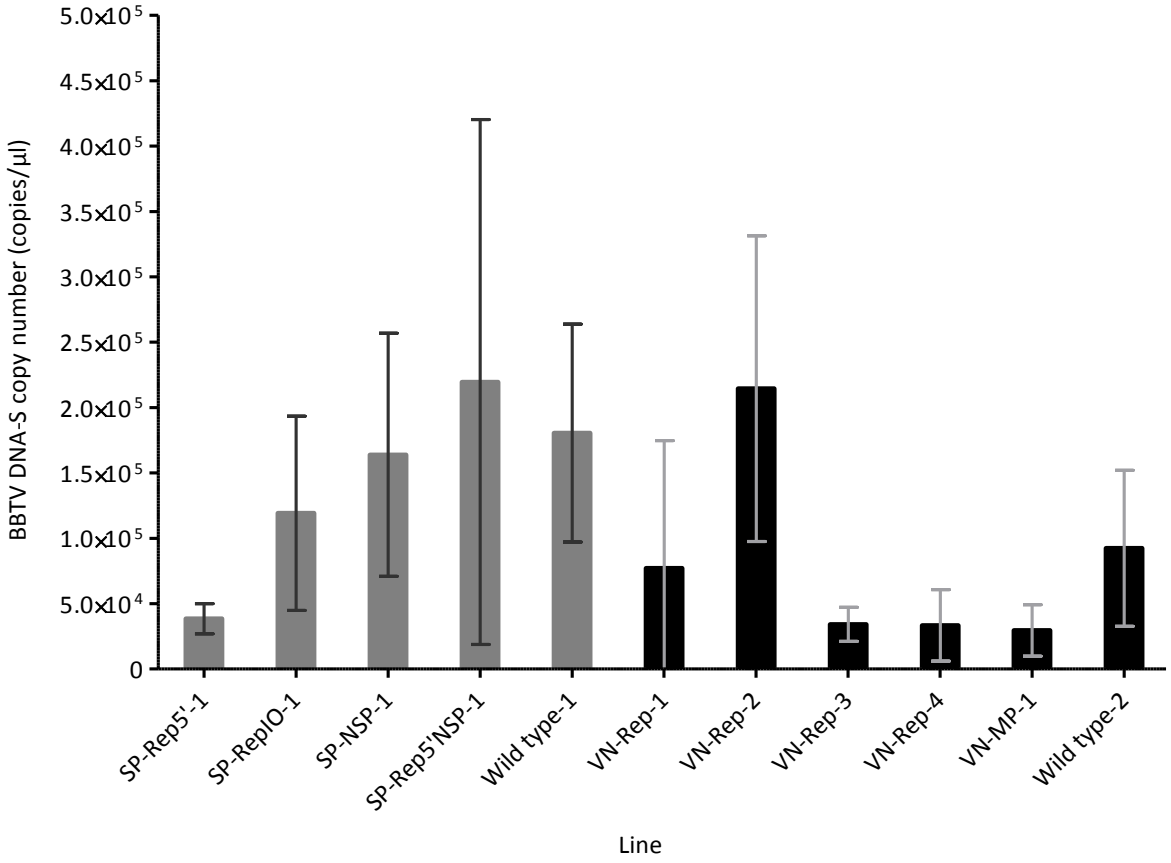


Figure 4.5: Quantification of BBTV DNA-S in wild-type and transgenic lines from glasshouse experiment 1. Transgenic lines were inoculated in two separate groups represented by grey bars (group 1) and black bars (group 2). The error bars represent standard deviation from the mean of absolute BBTV DNA-S copy numbers in 3 replicates of each transgenic line and wild type controls.

4.3.4 Detection of siRNAs

The presence of BBTV DNA-M-specific siRNAs in the resistant line SP-MP-2 from glasshouse experiment 1 was assessed by northern blotting. Total RNA was extracted from a BBTV-inoculated wild-type plant, in addition to two BBTV-inoculated and two non-inoculated SP-MP-2 plants, blotted onto nylon and hybridized with a P^{32}-labelled BBTV DNA-M-specific RNA probe. Several DNA-M-specific bands of the sizes expected for hairpin-derived siRNAs (21-24 nt) were detected in both the two inoculated and two non-inoculated SP-MP-2 transgenic plants as well as the inoculated wild-type control plant (Fig 4.6A). These sizes were estimated by comparison with the position of 21 nt miRNA159 that was present on blots that had been stripped and re-hybridised with a miRNA159-specific probe (Fig. 4.6B).

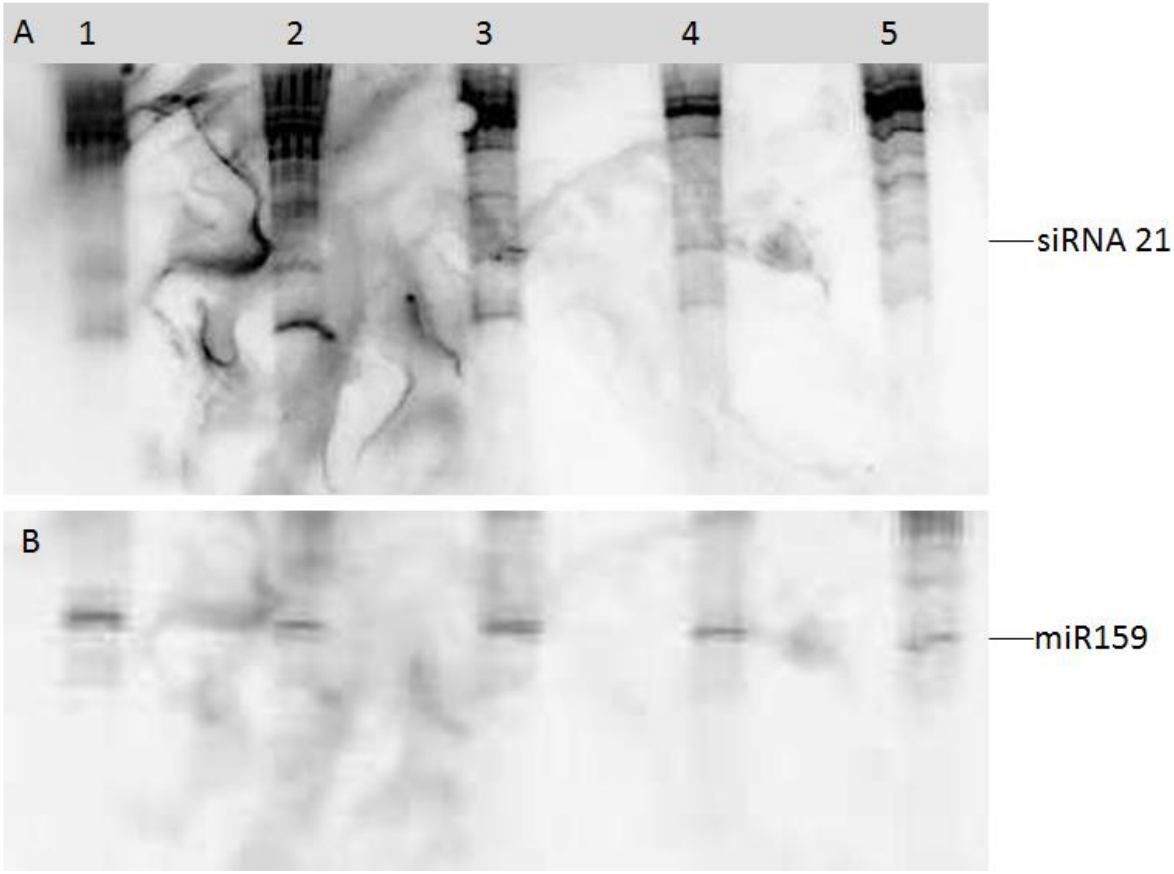


Figure 4.6: Detection of BBTV DNA-M-specific siRNAs in transgenic banana line MP-SP-2 by northern analysis. A) Extracts from a BBTV-infected wild-type banana (lane 1), two non-inoculated SP-MP-2 transgenic bananas (lanes 2 and 3) and two BBTV-inoculated SP-MP-2 transgenic bananas hybridised with a BBTV DNA-M-specific probe; and B) Re-probing of blot shown in 'A' with a miRNA159-specific hybridisation probe.

4.4 **Discussion**

Following their molecular characterization in Chapter 4, independently transformed transgenic banana lines expressing a range of hairpin cassettes prepared using BBTV DNA sequences were assessed for resistance to BBTV following inoculation using viruliferous aphids. Inoculation trials were staggered as plants were available at different times based on the transformation experiments as well as growth in tissue culture and also due to the availability of viruliferous aphids.

In glasshouse experiment 1, ten independent transgenic Dwarf Cavendish lines expressing a range of constructs were assessed for their response to BBTV inoculation. Non-transgenic Dwarf Cavendish control plants had a disease incidence ranging from 75-100%, while plants from 9/10 of the transgenic lines also developed characteristic BBTV symptoms and showed a disease incidence ranging from 50-100%, with BBTV infection confirmed by PCR. However, line SP-MP-2 transformed with the SP-MP hairpin cassette remained symptomless and virus-free for 10 months post-inoculation. Similarly, in glasshouse experiment 2, twenty three transgenic Dwarf Cavendish lines expressing either the SP-MP or SP-Rep5'MP constructs were assessed for their response to BBTV infection. The non-transgenic dwarf Cavendish control plants inoculated in group one and two had 100% infection rate at 12 weeks post-inoculation. However, only 1/12 wild-type plants developed symptoms at 12 weeks post-inoculation in the third inoculation group. As such, transgenic lines in this group were allowed more time in the glasshouse to develop symptoms. Of the 23 transgenic lines tested, six developed typical bunchy top disease symptoms with a final disease incidence ranging from 8 to 25% and PCR confirmed the presence of BBTV in all symptomatic plants.

The transgenic lines exhibited a varied response to inoculation with BBTV. For instance, the transgenic lines SP-MP-2 and -13, SP-Rep5'MP-6 and SP-Rep5'MP-13 which were previously shown to contain a single copy of the hairpin cassette remained disease free following the glasshouse inoculation trials. This finding is in line with the findings of Yang *et al.* (2014) who showed that lines with a single copy of an RNAi construct against the geminivirus, *Ageratum yellow vein virus*, were resistant to virus infection. In contrast, lines SP-NSP-1, SP-Rep5'NSP-1 and -2, SP-Rep5'-1, SP-RepIO-1, SP-MP lines-10, -11, -12 and -15 and VN-Rep1-4 in the present study, which were shown to have 2 to 6 copies of the respective hairpin

cassette, were all found to be susceptible to BBTV. Interestingly, Zhang *et al.* (2013) reported that transgenic plants with more than one copy of an RNAi construct targeting MDMV had an intermediate level of virus resistance, or were susceptible. In the current study, this trend was not absolutely consistent as some of the multi-copy lines transformed with the SP-MP and SP-Rep5'MP constructs, remained virus free during the screening period. It is important to note, however, that these lines were only tested once in the second glasshouse screening experiment and, as such, repeat challenges are required to confirm their resistance to BBTV infection.

The VN lines (VN-Rep-1-4 and VN-MP-1) tested in experiment 1 were prepared using DNA sequences from a Vietnamese BBTV isolate (in the Asian subgroup of worldwide BBTV isolates). The aim here was to assess whether the three BBTV target sequences were capable of generating a broad spectrum resistance, and so these lines were screened using the Australian BBTV isolate. The similarity between the VN and Australian BBTV isolates of DNA-N and DNA-R is between 85.5 to 90%, respectively (Karan *et al.*, 1994). When the VN lines were screened, disease incidences ranged on average from 58 to 100%, indicating that they were not resistant to the Australian BBTV isolate. These lines were shown to have between 2 to 6 copies of the respective transgene. It is possible that the presence of multiple copies of the hairpin in these plants may have reduced the chances of obtaining a resistant line. Alternatively, this result may be due to the lower level of sequence homology between the transgene sequences and the virus isolate used for challenge. In previous studies, a sequence difference of more than 15% was enough to significantly reduce the effectiveness of the RNAi response against the geminivirus pepper golden mosaic virus (Medina-Hernández *et al.*, 2013), with an optimal response obtained using RNAi constructs with a nucleotide sequence identity of at least 85% (Benedito *et al.*, 2004). Although nucleotide sequence similarity of DNA-R and DNA-N (90% and 85.5%, respectively) between SP and VN isolates (Karan *et al.*, 1994) meets the requirement for effective silencing by heterologous RNAi constructs from the two components, the current study only tested a small number of lines for these constructs. Thus, there is a need to generate single copy and more multi-copy transgenic lines with the VN-Rep, VN-NSP and VN-MP constructs to confirm this result or demonstrate broad-range resistance.

When plants from glasshouse experiment 1 were tested by rtPCR to determine the level of BBTV DNA present, the transgenic plants had levels of BBTV which were not significantly different from the non-transgenic controls, except for line SP-Rep5'-1. This line was not considered resistant or even tolerant based on the results of the inoculation trials with an average disease incidence of 83%. It is unclear why this line had lower levels of BBTV DNA. However, when compared with the controls from the second inoculation group in experiment 1, the level of BBTV DNA was not significantly different. Interestingly, the controls from the second inoculation group had an average level of BBTV DNA which was about half of the first inoculation group. Although not statistically significant, this result suggests that the levels of virus DNA in wild type plants can vary and might be related to the onset of infection and the time of sampling.

When plants from the resistant line SP-MP-2 were assessed by northern blotting, the presence of siRNAs was detected relative to the 21 bp size of the miR159 control signal. In the transgenic plants tested, MP-specific siRNAs were detected at a range of sizes consistent with the miRNA159 control. Different siRNA sizes ranging from 21-24 have been reported from transgenic as well as non-transgenic plants (Maruthi *et al.*, 2014; Montes *et al.*, 2014) and the results of this study are consistent with these previous reports. MP-specific siRNAs were also detected in the non-transgenic Dwarf Cavendish BBTV-infected control plant, indicating that a functional RNAi system is present in banana plants. However, since these wild-type plants are highly susceptible to BBTV, it may be that BBTV is able to overcome this defense mechanism in this cultivar.

Based on the results of inoculation experiments described in this chapter, several constructs appear to have conferred resistance to BBTV in Dwarf Cavendish bananas. The resistant lines developed in this study should therefore undergo further assessment under field conditions to confirm transgene and resistance stability and observe any negative phenotypic changes to the plant or to its agronomic traits. A substantial number of additional lines are also available for further glasshouse assessment which, based on these results, would be highly likely to increase the number of potentially resistant lines.

CHAPTER 5

DEVELOPMENT OF A METHOD FOR THE MECHANICAL INOCULATION OF BBTV

5.1 Introduction

Plant virus infectious clones have become essential molecular tools in determining the functions of viral genes, assessing their role in replication, pathogenesis and transmission as well as challenging plants for virus resistance. However, efficient virus inoculation strategies are necessary to achieve this. In begomoviruses and other members of the family *Geminiviridae*, agro-inoculation is widely applied as an efficient means of transferring binary vectors harbouring viral clones into host plants (Elmer *et al.*, 1988; Grimsley *et al.*, 1987; Trenado *et al.*, 2011). Past studies have shown that a clone containing a single genome copy is sufficient for successful infection by certain begomoviruses (Bonilla-Ramírez *et al.*, 1997; Lapidot *et al.*, 2007; Stanley & Townsend, 1986; Unseld *et al.*, 2004). However, an increase in infectivity was found when clones with tandem repeats of the virus genome were used (Bonilla-Ramírez *et al.*, 1997; Elmer *et al.*, 1988).

Recently, a simple method for the construction of infectious clones of begomoviruses has been demonstrated by partial digestion of rolling circle amplification (RCA)-amplified virus DNA and subsequent cloning of dimeric forms of the genome into binary vectors (Ferreira *et al.*, 2008; Wu *et al.*, 2008). Similarly, work by Sicard *et al.* (2013) demonstrated the possibility of inducing virus infection in host plants using a mixture of cloned virus DNA components from the multipartite nanovirus *Faba bean necrotic stunt virus* (FBNSV). In order for virus infection to occur when using multipartite plant viruses, it is assumed that at least one copy of each of the integral genomic components of the virus must be present in one cell at the same time (Grigoras *et al.*, 2009; Iranzo & Manrubia, 2012; Sicard *et al.*, 2013).

A number of techniques have been successfully used for the mechanical inoculation of plant virus infectious clones. These include sap injection, DNA particle bombardment, grafting and *Agrobacterium*-mediated inoculation (agro-inoculation), with the latter now widely used and considered the method of choice in amenable hosts (Al Abdallat *et al.*, 2010; Malik *et al.*, 2011; Wu *et al.*, 2008).

Despite previous success and the general availability of techniques for generating and delivery of infectious clones of DNA viruses, to date there are no reports of the successful inoculation of banana with cloned copies of the *Banana bunchy top virus* (BBTV) genome. BBTV has a multipartite, single-stranded DNA genome comprising at least six separate components. Whether all of these components are required to initiate infection remains to be demonstrated. Recently, there was a report of infection initiated using agro-inoculation of a clone of the *Banana streak* MY *virus* (BSMYV) genome in bananas (Bjartan, 2012). This would suggest that artificial inoculation of bananas using a plant virus infectious clone is indeed possible. Therefore, this chapter aimed to investigate a method for the mechanical inoculation of bananas with BBTV.

The specific objectives of this chapter were to:

1. Generate BBTV DNA component constructs for mechanical inoculation of banana
2. Evaluate the Helios Gene Gun system for mechanical inoculation of BBTV in banana
3. Evaluate agro-inoculation of BBTV in banana

5.2 Materials and methods

5.2.1 Source plant materials and plasmids

BBTV- and BSMYV-infected banana plants were maintained in a plant growth chamber at 28°C. *Tobacco yellow dwarf virus* (TYDV) and *Tomato yellow leaf curl virus* (TYLCV)-infected tobacco (*Nicotiana tobacum*) plants were kindly provided by Dr Ben Dugdale (QUT) and maintained at 23°C. Tissue cultured banana plantlets were acclimatised and maintained for inoculation experiments as described in section 4.2.2 and tobacco plants (cv. Samsun) were raised from seed.

A plasmid vector containing the constitutive *Cauliflower mosaic virus* (CaMV) 35S promoter driving the *β*-glucuronidase (GUS) gene (pBIN-35S-GUS) and plasmid DNA constructs containing infectious clones of TYLCV or TYDV were provided by Dr Ben Dugdale (QUT), while the BSMYV infectious clone was provided by Dr Anthony James (QUT).

5.2.2 Rolling circle amplification

Total nucleic acid (TNA) was extracted from plants as described in section 2.5.1. RCA was carried out using the Illustra Templiphi kit (GE Healthcare, United Kingdom) as follows: 5 µL of sample buffer was mixed with 1 µL of TNA and incubated at 95°C for 3 min. The mixture was immediately chilled on ice, followed by the addition of 4 µL of premixed reaction buffer/enzyme mix. The reaction mix was incubated at 30°C for 18 h and the enzyme denatured by incubating at 65°C for 10 min.

5.2.3 Cloning of BBTV genomic components

Published full-length genome sequences of BBTV were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/) and analysed using the Vector NTI suite v.11 software package (Life Technologies, Australia) to identify unique restriction sites useful for preparing genome-length fragments (monomers and dimers) from RCA-amplified DNA. RCA-amplified TNA derived from BBTV-infected bananas was subjected to restriction enzyme digestion using *Stu*I, *Spe*I, *Sca*I, *Kpn*I, *Mfe*I and *Pac*I, which were predicted to cleave BBTV DNA-R, -U3, -S, -M, -C and –N, respectively, at a single site.

Following digestion of the BBTV RCA products using component-specific restriction endonucleases, the resulting linear DNA was gel electrophoresed, excised and purified (section 2.4.4), and self-ligated (section 2.4.6) to regenerate circular DNA molecules. These re-circularised forms of each BBTV DNA component were then used as a template for subsequent RCA (see below) as well as PCR using component-specific, outward-facing (i.e. back-to-back) PCR primers flanked by unique restriction sites (Table 5.1). The restriction sites engineered into the component-specific PCR primers were designed to allow for digestion of PCR-amplified DNA components and their subsequent ligation into a binary vector for plant transformation. PCR was carried out as described in section 2.4.2 and the resulting PCR products were excised, purified (section 2.4.4) and ligated into pGemT-Easy (Promega Corp, USA). Recombinant plasmid DNAs were isolated as described in section 2.4.10 and verified by sequencing (2.4.12). Full-length BBTV DNA components were digested using restriction sites engineered into the primers and subsequently ligated into appropriately digested pOPT-EBX (section 2.7.1). Clones were isolated and sequenced as previously described.

Table 5.1: Outward-facing primers used for the amplification of full-length BBTV genomic DNA components

Target BBTV component	Virion sense primer (5` to 3`)	Complementary sense primer (5` to 3`)
DNA-R	GCcccgggTTAGTGCGCCACGTA	GCtgtacaACATCTGATCTCGCCGT
DNA-U3	GCtgtacaGGCGCTGGGGCTTATTATT	GCacgcgtTACGTCAGCAGAAGTTCATTC
DNA-S	GCacgcgtTTAGTGCGCCACGTA	GCgttaacACATCTGGCCCACCACTAA
DNA-M	GCcccgggTTAGTGCGCCACGTAAGCGC	GCtgtacaACATCTGGCCCACCACTAAAG
DNA-C	GCtgtacaTTAGTGCGCCACGTAAGC	GCacgcgtACATGGACCCTACGTTGCC
DNA-N	GCacgcgtACGGGACATGACGTCAGC	GCgttaacCACGGGGGGTAATAATAG

Note: The restriction sites for *Sma*I (cccggg), *Bsr*GI (tgtaca), *Mlu*I (acgcgt) and *Hpa*I (gttaac) included in primer sequences are indicated in lower case.

In addition to the PCR-derived full-length BBTV DNA components described above, a set of BBTV DNA dimeric components was also prepared. Using the self-ligated RCA-derived BBTV DNAs prepared earlier, a second round of RCA was employed using each individual BBTV DNA component preparation. These amplification products were subjected to partial restriction endonuclease digestion using the enzymes described previously to generate dimers of each BBTV DNA component. The resulting BBTV DNA component dimers were subsequently ligated into pOPT-EBX cleaved using *Stu*I, *Spe*I, *Sca*I, *Kpn*I, *Mfe*I or *Pac*I, respectively.

5.2.4 Mechanical inoculation of plants using the Helios Gene Gun system

Gold particles were coated with DNA according to the method described by Woods and Zito (2008) with minor modifications. Briefly, 6-8 mg of 1.6 µm diameter gold particles were mixed with 100 µL of 0.1 M spermidine and subjected to sonication at room temperature for 20 s. Twenty micrograms of RCA-amplified DNA was added to the gold/spermidine mixture, followed by 100 µL of 1 M $CaCl_2$. The mixture was vortexed and incubated at room temperature for 10 min to allow for DNA precipitation onto the gold particles. The particles were then washed three times using 100% ethanol and finally re-suspended in 3.2 mL of ethanol supplemented with 0.14 mg of PVP. Gold particles were coated onto plastic tubing and bombarded into test plants according to the manufacturer's instructions (Bio-Rad). Plants were inoculated by bombarding the three youngest leaves, with the first fully expanded leaf considered the youngest when inoculating bananas.

5.2.5 *Agrobacterium*-mediated inoculation of banana plants

Agrobacterium tumefaciens strain AGL1 cells were transformed by electroporation as described in section 2.4.8. Following PCR confirmation of the correct vector DNA, overnight bacterial cultures were grown as described in section 2.4.8 and the bacterial cells were pelleted by centrifugation at 1000 x *g* for 10 min. The pellet was re-suspended in 10 mL of antibiotic-free MMA media containing 200 µM acetosyringone and incubated at room temperature on a shaker at 70 rpm for 3 h. The OD_{600} was then adjusted to 0.5 and banana plants were inoculated by needle injection. Briefly, a 1 mL syringe was filled with agrobacterium cells and used for injection of the pseudostem directly above the corm region. For each inoculation experiment, the BSMYV infectious clone was included as a control.

5.2.6 GUS histochemical assays

Plant tissues were immersed in a GUS substrate solution of 0.5 M tri-sodium citrate (pH 7.0), 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, 0.1% TritonX-100 and 250 mg/L 5-Bromo-4-chloro-3-indolyl-β-D-glucuronic acid. Banana leaf and pseudostem tissues were sampled 3 days post-bombardment and immersed in GUS substrate, vacuum infiltrated for 1 h, then incubated overnight at 37°C. Tissues were cleared in 70% ethanol and GUS expression estimated by counting the number of blue foci observed and averaging these totals.

5.2.7 Assessment of inoculated plants

Following delivery using the Helios Gene Gun or *Agrobacterium*, inoculated and non-inoculated control plants were maintained in plant growth chambers at either 28°C for bananas or 23°C for tobacco. All plants were visually monitored for symptom development over a 12 week period and leaf samples were collected for an end-point PCR assay at 12 weeks post-inoculation. Disease incidence was determined by recording the number of replicates displaying symptoms of viral infection, expressed as a percentage of the total number of replicates inoculated. Each virus inoculation experiment was carried out three times and the average disease incidence across the three repeat experiments was subjected to analysis of variance. PCR detection of each virus was carried out as described in section 2.4.2 using virus-specific primers: for BBTV, primers were BBTV-C-fwd 5` GTTCTCTTGGAGTGATTGTCAG 3` and BBTV-C-rev 5`CTTAATCTCTCTCTTGACATCG 3`; for BSMYV, primers were MYS-F1 5` TAAAAGCACAGCTCAGAACAAACC 3` and MYS-R1 5` CTCCGTGATTTCTTCGTGGTC 3` (Geering et al., 2000); for TYDV, primers were TYDV-Rep-F 5`ATGCCTTCAGCCCCCCAGAAAACC 3` and TYDV-Rep-R 5` TCAGTGACTCGACGATTCAGGAGC 3`; and for TYLCV, primers were TYLCV-Rep-F 5` GCGATCGCATGCCTCGTTTATTTAAAATA TATGCC and TYLCV-Rep-R 5` GCGAGCTCTTACGCCTTATTGGTTTCTTCTTGGC 3`.

5.3 Results

5.3.1 Preparation of inoculum

5.3.1.1 Virus DNA prepared using RCA

Three different types of BBTV inoculum were assessed for infectivity. The first involved the direct use of high molecular weight concatameric preparations of BBTV generated using RCA, while the second and third types of inoculum were BBTV monomers and dimers that were cloned using the RCA products.

The high molecular weight concatameric preparations of BBTV were generated by RCA from total nucleic extracted from BBTV-infected plants. As controls, similar preparations were generated from BSMYV-, TYDV- and TYLCV-infected plants. To prepare the BBTV monomers, RCA-amplified BBTV DNAs were digested using *Stu*I, *Spe*I, *Sca*I, *Kpn*I, *Mfe*I and *Pac*I, in separate reactions resulting in ~1.1 kb linear monomeric forms of each of the six known BBTV DNAs (Fig 5.1). The six BBTV DNAs were visualised on agarose gels, purified and self-ligated, then re-amplified by RCA to increase the quantity of virus DNA (relative to background DNA) in preparations.

The self-ligated, re-amplified RCA products were subjected to PCR using outward-facing primers (Table 5.1; Fig 5.2). The amplicons were cloned into pGemT-Easy and their integrity confirmed by PCR and sequencing. Sequence analysis identified a number of nucleotide mismatches in each virus DNA, however, a functional protein-coding sequence was maintained in each. The full-length PCR-amplified BBTV DNAs were subsequently excised using specific restriction enzymes engineered into the primer sequences (Table 5.1) and ligated into appropriately digested pOPT-EBX. Recombinant pOPT-EBX vectors containing the six BBTV DNAs were identified by restriction digestion (Fig 5.3) and were electroporated into *A. tumefaciens* strain AGL1. Individual AGL1 cultures of each BBTV DNA component cloned into pOPT-EBX were prepared, adjusted to an equivalent optical density and mixed in equal amounts prior to agro-inoculation of banana plants.

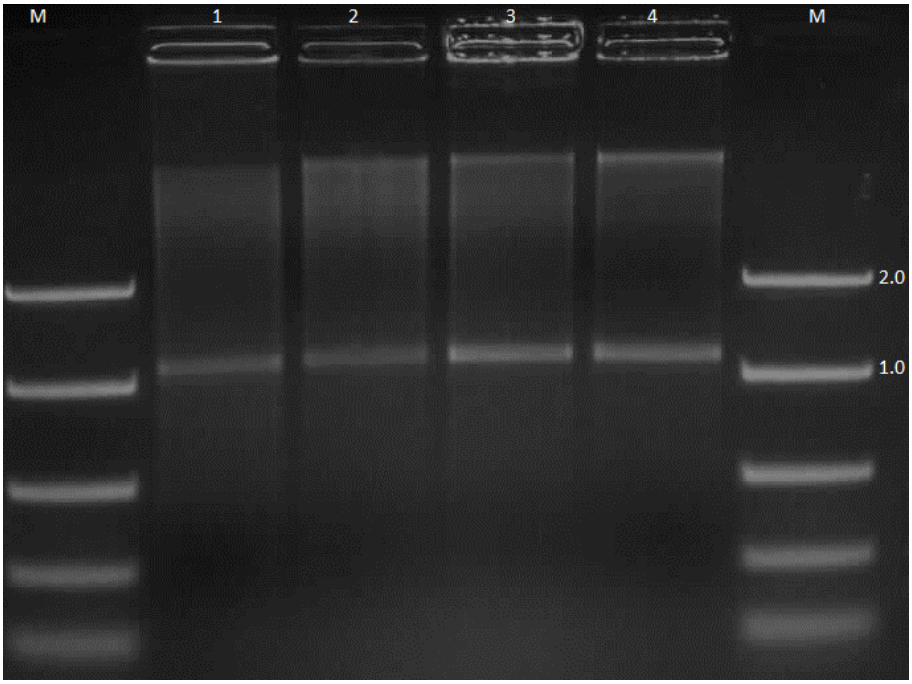


Figure 5.1: Agarose gel electrophoresis of RCA-amplified BBTV DNA-R, -U3, -S and –M digested with *Stu*I, *Spe*I, *Sca*I and *Kpn*I, respectively. Lanes 1-4 represent BBTV DNA-R, -U3, -S and –M. Digest products of ~1.1 kb are present representing linearized, full-length monomeric DNA of each component. M is EasyLadder 1 (Bioline). Digest results for DNA-C and DNA-N were done separately and are presented in Appendix 2.

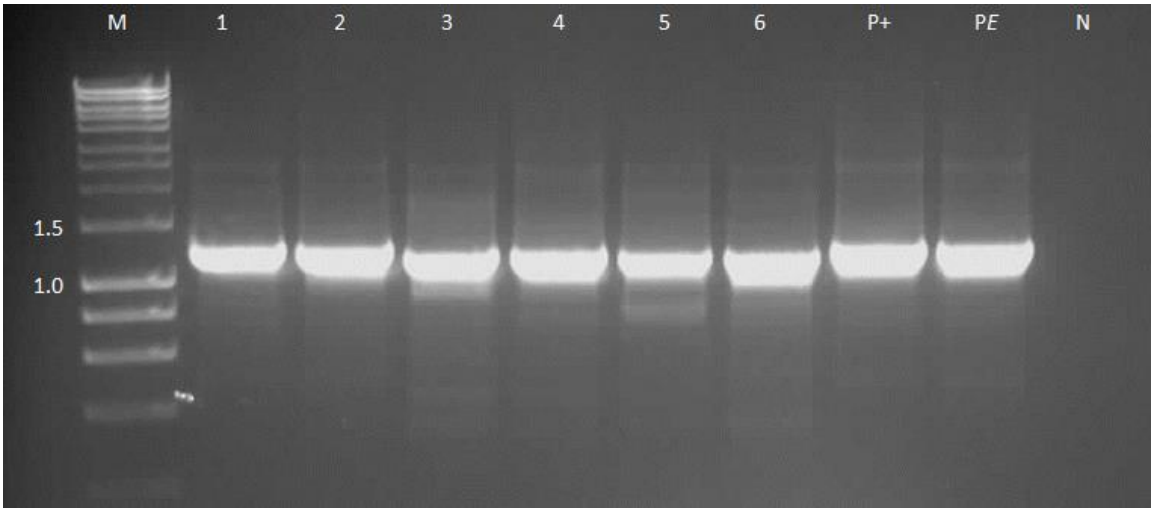


Figure 5.2: Agarose gel electrophoresis of PCR amplicons of BBTV monomer inserts in pGem-T Easy backbone generated using M13 primers. PCR products of ~1.1 kb are present representing genome length of the respective BBTV component inserts in pGem-T Easy. Lanes 1-6 represent BBTV DNA-R, -U3, -S, -M, -C and –N, respectively, P+ is purified plasmid control, PE is *E. coli* containing the plasmid control and N is the no template control. The expected PCR product size was 1.1 kb. M is HyperLadder 1 (Bioline).

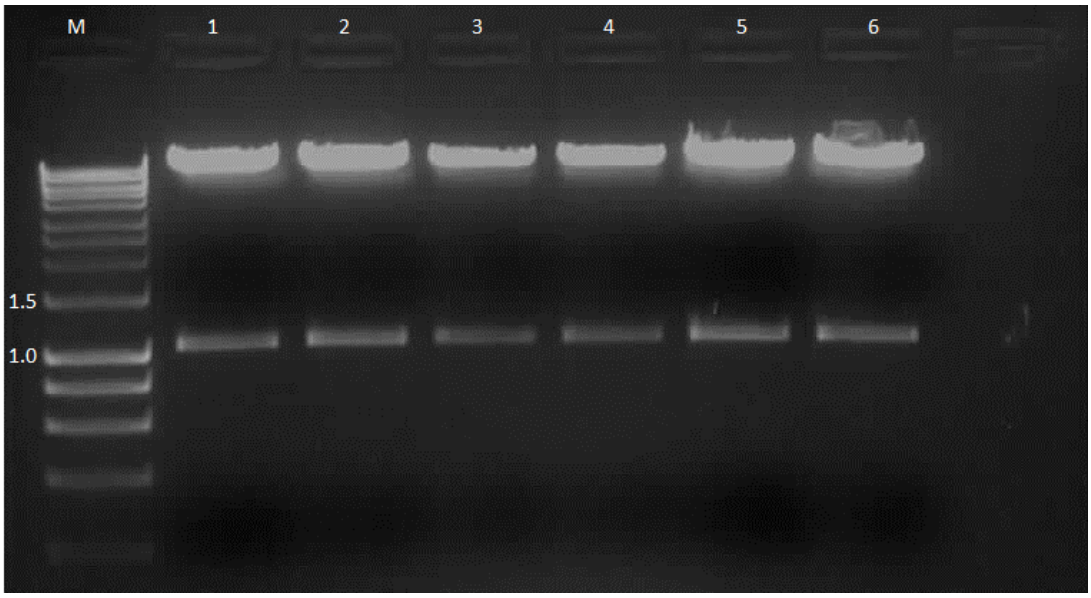


Figure 5.3: Agarose gel electrophoresis of digest products of BBTV genome length inserts in pOPT-EBX binary vector. Lanes 1-6 represent BBTV DNA-R, -U3, -S, -M, -C and –N respectively. Digest products of ~1.1 kb are present representing linearized, full-length DNAs of each component inserts in pOPT-EBX binary vector. M is HyperLadder 1 (Bioline).

To prepare dimers of each BBTV DNA, RCA-amplified BBTV DNAs were subject to partial digestion using the enzymes *Stu*I, *Spe*I, *Sca*I, *Kpn*I, *Mfe*I and *Pac*I described previously in 5.3.1 and then ligated into appropriately digested pOPT-EBX resulting in six distinct binary vectors. The presence of the dimers in the binary vectors was confirmed by restriction digests (Fig 5.4).

5.3.2 Optimisation of bombardment parameters for optimal gene expression using the Helios gene gun system

Optimisation of the Helios Gene Gun system for delivering DNA into banana plants was carried out using a vector expressing the GUS reporter gene (pBin-35S-GUS). Plasmid DNA was coated onto gold particles and delivered into the adaxial surface of banana leaf, as well as the outer leaf sheaths comprising the pseudostem. To determine the effect of shooting pressure and distance from the plant tissue surface on reporter gene expression, helium pressures of 200, 250, 300, 350 and 400 psi and distances ranging from 0, 0.5 and 1.0 cm were tested. In all treatments, three separate bombardments were carried out onto the same tissue target area. GUS expression was estimated using GUS histochemical assays with plant tissues three days post-bombardment and calculating the average number of blue foci observed.

Using a pressure of 200 psi and a distance of 0.5 cm, no visual damage to the tissue occurred and the average number of blue foci observed, three days post-bombardment, was 18.7 (Fig. 5.5 and 5.6a). Similarly, when the pressure was increased to 250 psi (at a distance of 0.5 cm), the tissue remained undamaged but the average number of blue foci increased to 59.7 (Fig. 5.6b). Further increases in pressure up to 400 psi at the same distance resulted in increased damage to the leaf surface (Fig. 5.6c, d, e) and a subsequent decrease in GUS expression (Fig. 5.6c). At pressures above 400 psi, the gold particles often completely penetrated through the leaf thus limiting GUS expression to just a small number of blue foci on the periphery of the bombarded area.

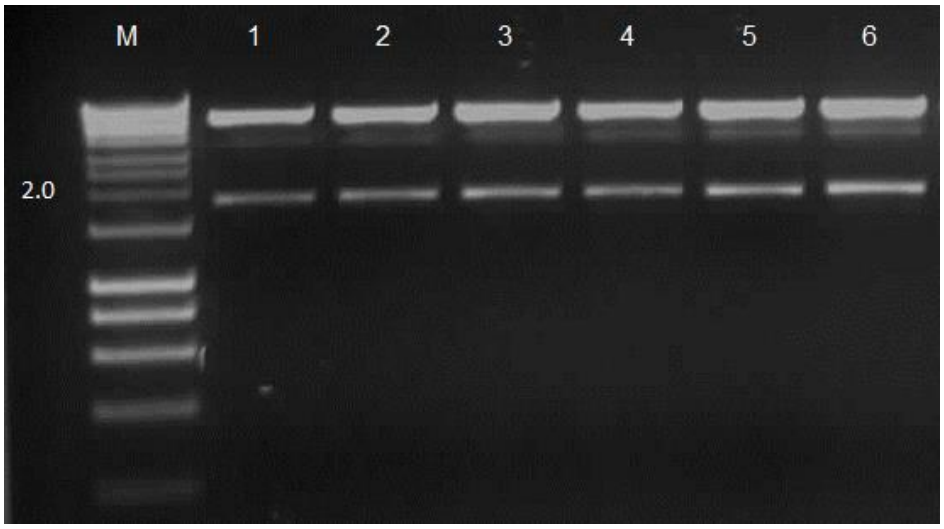


Figure 5.4: Agarose gel electrophoresis of digest products of dimeric genome length inserts in pOPT-EBX binary vector. Lanes 1-6 represent BBTV DNA-R, -U3, -S, -M, -C and -N. Digest products of ~2.0 kb are present representing linearized dimeric DNA of each component excised from the pOPT-EBX vector backbone. M is HyperLadder 1 (Bioline).

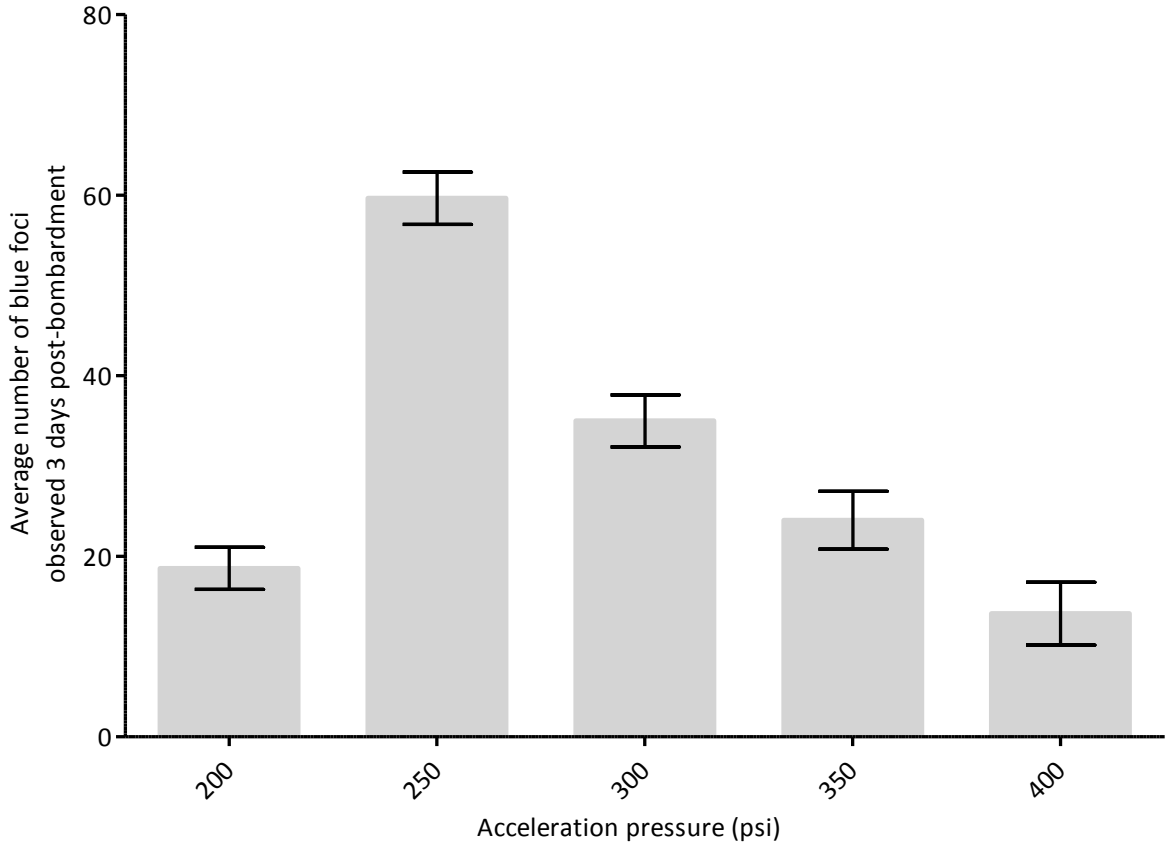


Figure 5.5: Effect of pressure on GUS expression in banana leaf tissue at a distance of 0.5 cm. The error bars represent standard deviation (SD) from the mean of GUS expression on three replicates of banana tissue.

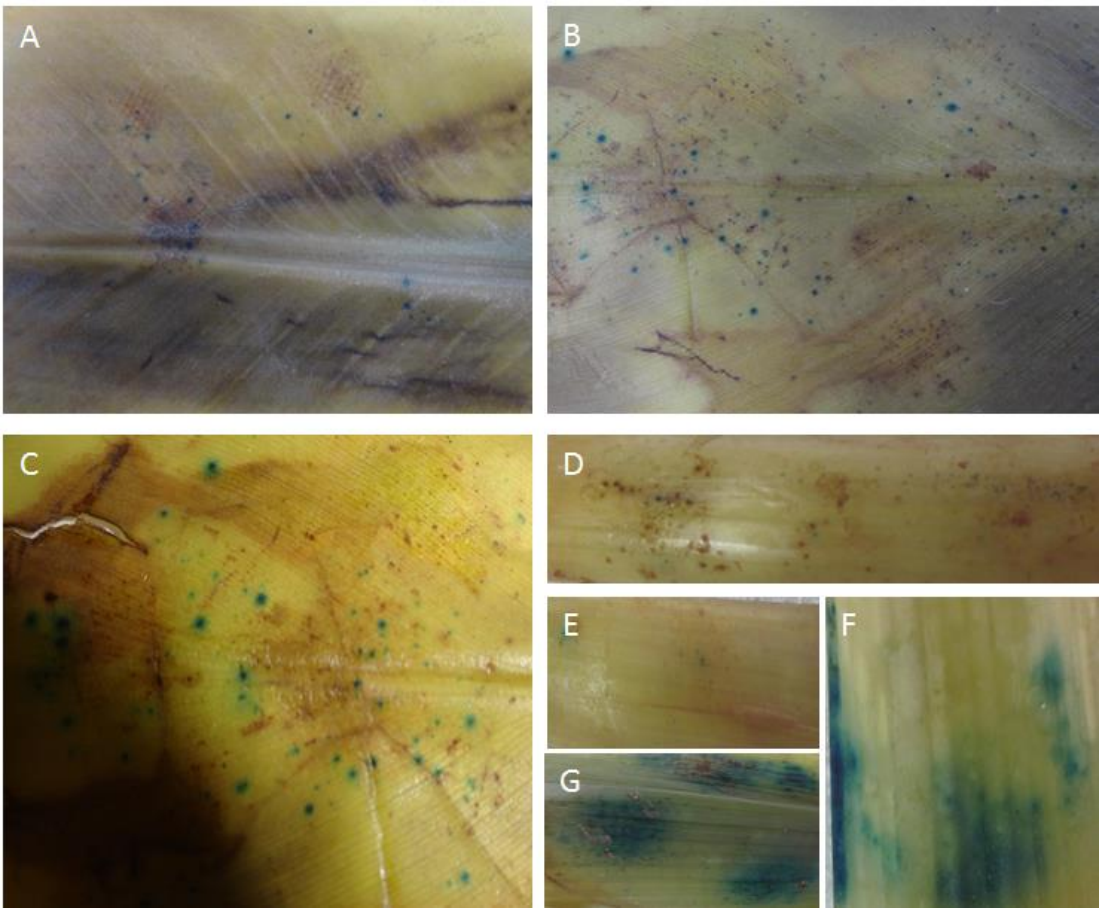


Figure 5.6: GUS expression in banana leaf and pseudostem tissue following bombardment using the Helios Gene Gun A) Leaf tissue bombarded at 0.5 cm and 200 psi, B) Leaf tissue bombarded at 0.5 cm and 250 psi, C) Leaf tissue bombarded at 0.5 cm and 300 psi, D) Pseudostem tissue bombarded at 0.5 cm and 200 psi, E) Pseudostem tissue bombarded at 0.5 cm and 250 psi, F) Pseudostem tissue bombarded at 0.5 cm and 300 psi and G) Pseudostem tissue bombarded at 0.5 cm and 350 psi.

Following the bombardment of leaf tissues at various acceleration pressures and a distance of 0.5 cm, the effect of particle acceleration pressures on reporter gene expression at shooting distances of 0 and 1 cm was investigated. At a distance of 0 cm from the leaf surface, bombardment using pressures of 200, 250 and 300 psi resulted in an average of 20, 51.7 and 15.3 blue foci, respectively, while at a distance of 1 cm, pressures of 200, 250 and 300 psi resulted in an average of 6, 43.7 and 24.3 blue foci, respectively (Fig. 5.7). At all distances tested, a bombardment pressure of 250 psi resulted in higher average GUS expression, with the highest average expression (59.7 foci) observed when tissue was bombarded at a distance of 0.5 cm. Based on these results, bombardment parameters of 250 psi at a distance of 0.5 cm from the tissue surface were used for all subsequent bombardments of DNA coated gold particles into banana leaf tissue.

Banana pseudostem tissues were also tested at a range of pressures (at a distance of 0.5cm) to determine the optimum pressure for bombardment. However, accurate counting of blue foci was complicated by diffusion of the stain as expression intensity increased (Fig. 5.6f & g). As a result, direct quantitative determination of GUS expression was not possible. Generally, it was observed that a pressure of 200 psi (Fig. 5.6d) had the lowest level of average GUS expression in pseudostem tissue, with a slight increase in the number of blue foci when the pressure was increased to 250 (Fig. 5.6e) and a significantly greater increase seen when the pressure was increased to 300 psi (Fig. 5.6f). Although the level of GUS expression at 350 psi was approximately equal to 300 psi (Fig. 5.6g), a greater level of tissue damage was observed at 350 psi and so the lower of the two pressures was considered preferable. Therefore, a pressure of 300 psi and a distance of 0.5 cm were considered optimum for bombardment of banana pseudostem.

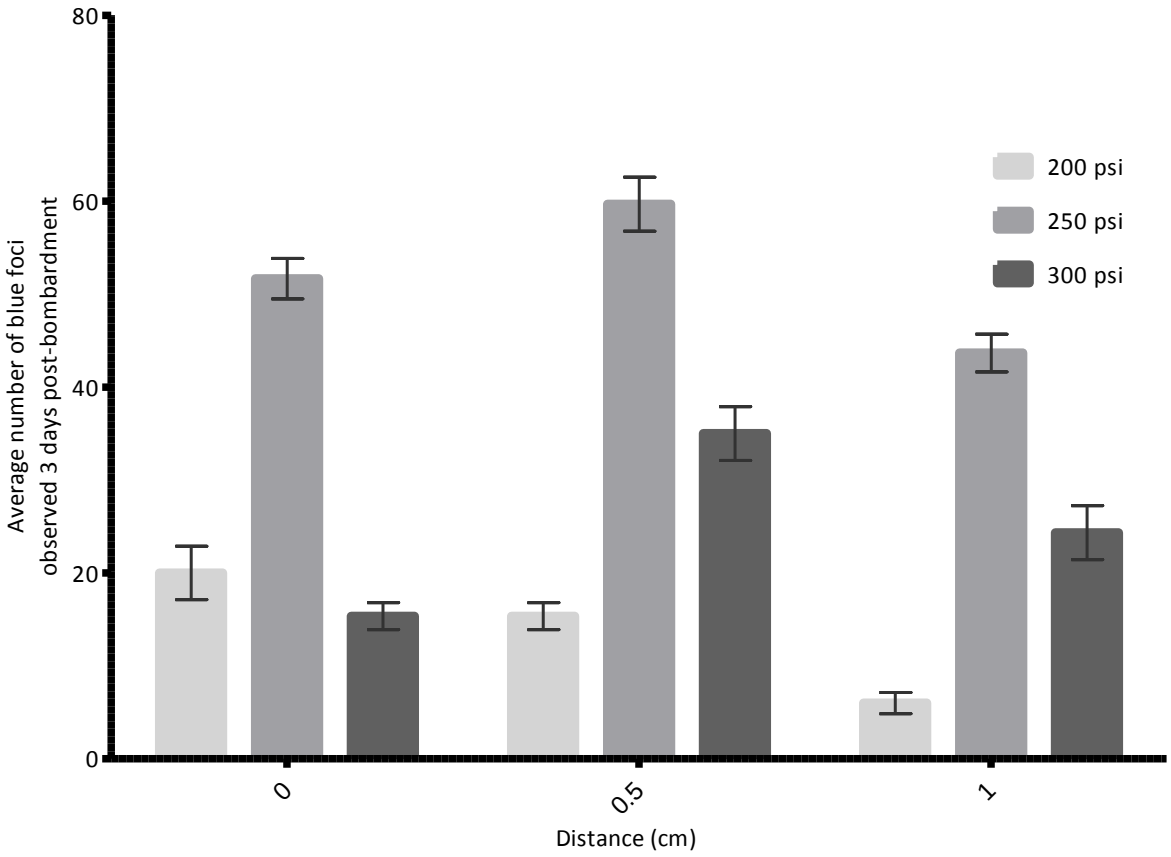


Figure 5.7: Effect of distance on average GUS expression in banana leaf tissue. The error bars represent standard deviation (SD) from the mean of GUS expression on three replicates of banana tissue.

Following the optimisation of the Helios Gene Gun system for delivery of DNA into banana tissues, the system was re-evaluated for tobacco leaf tissue. Bombardment of tobacco leaves was carried out at pressures ranging from 180 to 250 psi at a distance of 0.5 cm. At a pressure greater than 200 psi, the leaf surface was found to be severely damaged. However, when gold particles were bombarded at a pressure of 180 psi there was little penetration of the gold particles into the leaf tissues. As a result, the optimised bombardment conditions for delivery of gold particles into tobacco leaf tissue were a pressure of 200 psi and a distance of 0.5 cm.

5.3.3 Mechanical inoculation of RCA-amplified DNA using the Helios Gene Gun

In this strategy, concatameric viral DNA, produced by RCA using total nucleic acid extracted from virus-infected plants was used as the inoculum for artificial infection. The RCA-amplified virus DNA was coated onto gold particles and inoculated into banana leaves using the Helios Gene Gun and the optimised conditions described above. As controls, banana leaves were inoculated using RCA-amplified BSMYV DNA, while tobacco plants were inoculated using RCA-amplified TYDV and TYLCV.

Following biolistic delivery of the RCA-amplified DNAs, plants were monitored for symptom development for 12 weeks and then tested by PCR using virus-specific primers. Each bombardment assessment was carried out in three independent trials, with at least 24 plants bombarded for all treatments in each trial.

When leaves of tobacco plants were bombarded with RCA-amplified DNA from TYLCV-infected tobacco, an average of 50% of plants inoculated developed characteristic disease symptoms including a downward curling of the leaf margins as well as vein-clearing and vein-thickening (Table 5.2, Fig 5.8). PCR analysis of plants inoculated in the first trial confirmed the presence of TYLCV in all plants which developed symptoms (Table 5.2, Fig 5.9), while no products were amplified from non-symptomatic plants. Non-inoculated plants were also maintained and upon analysis tested negative for TYLCV (Fig 5.9). Due to the high correlation between the results of PCR and symptoms in the first trial, tobacco plants included in trials 2 and 3 were not tested for virus presence by PCR.

Table 5.2: Disease incidence in tobacco and banana plants bombarded with a range of different viral inocula using the Helios Gene Gun

		Trial 1			Trial 2		Trial 3	
Virus	Inoculum	No. symptomatic	No. PCR +ve	Disease incidence (%)	No. symptomatic	Disease incidence (%)	No. symptomatic	Disease incidence (%)
TYLCV	RCA from TNA extract	12	12	50	10	42	14	58
TYDV	RCA from TNA extract	10	10	42	12	50	16	67
BSMYV	RCA from TNA extract	0	0	0	0	0	0	0
BBTV	RCA from TNA extract	0	0	0	0	0	0	0
BBTV	RCA from purified, self-ligated BBTV DNAs	0	0	0	0	0	0	0
TYLCV	Infectious clone/plasmid DNA	26	26	100	18	75	22	92
TYDV	Infectious clone/plasmid DNA	16	16	61	16	61	18	69
BSMYV	Infectious clone/plasmid DNA	0	0	0	0	0	0	0
BBTV	monomers from back to back primers	0	0	0	0	0	0	0

Figure 5.8: Tobacco plant bombarded with TYLCV RCA coated gold particles showing curling, raised leaf surface, on young newly formed leaves three weeks post-bombardment.

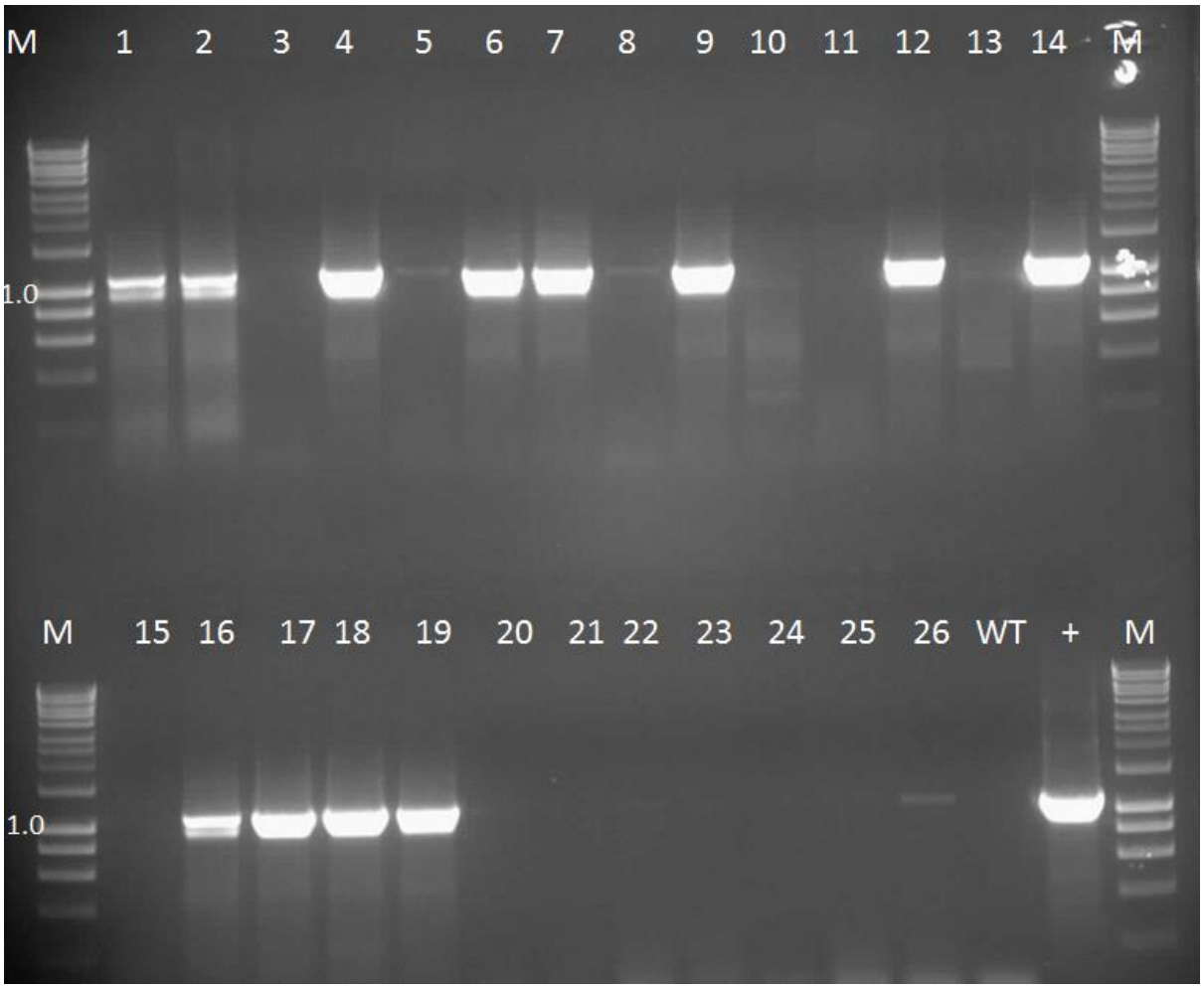


Figure 5.9: PCR screening for TYDV presence in tobacco plants mechanically inoculated with the TYDV infectious clone in trial 1. Lanes 1-14 and 15-26 represent 26 tobacco plants inoculated with the infectious clone, WT is non-inoculated control plant and + is positive control (plasmid DNA). The expected size of the PCR product is approximately 1 kb. M is the HyperLadder 1 (Bioline).

Similarly, when tobacco plants were bombarded with RCA-amplified DNA from TYDV-infected tobacco, an average of 53% of plants inoculated developed characteristic symptoms including stunting, dwarfing and yellowing (Table 5.2). PCR analysis again confirmed the presence of TYDV in all plants with symptoms, while non-inoculated controls tested negative (Table 5.2). As with TYLCV, plants from trials 2 and 3 were not tested by PCR due to high correlation between symptom and PCR results.

When banana leaves were bombarded with RCA-amplified DNA from BSMYV-infected plants, none of the plants developed typical symptoms and no plants tested positive for BSMYV DNA by PCR at 12 weeks post-inoculation. Similarly, when banana leaves were bombarded with RCA-amplified DNA from BBTV-infected plants, no plants developed typical symptoms and none were positive by PCR. Furthermore, none of the 24 banana plants inoculated using a mixture of the six purified BBTV DNAs which were re-amplified using RCA developed symptoms and no plants tested positive for BBTV (Table 5.2).

5.3.4 Mechanical inoculation of cloned virus genomes using the Helios Gene Gun

In this approach, attempts were made to mechanically inoculate bananas with BBTV using cloned full-length DNA of each BBTV component, mixed in equal ratios and co-bombarded into banana leaf tissue. As controls, banana plants were bombarded with an infectious clone of BSMYV while tobacco plants were bombarded with infectious clones of TYLCV and TYDV. A summary of the results is shown in Figure 5.10.

When tobacco leaf tissue was bombarded with gold particles coated with plasmid DNA of the TYLCV infectious clone, an average of 89% of target plants developed typical symptoms at 12 weeks post-inoculation (Table 5.2). The presence of TYLCV in all plants with symptoms was confirmed by PCR (Fig 5.11). Similarly, when tobacco leaves were bombarded with the TYDV infectious clone, an average of 63% of inoculated plants developed typical symptoms across the three trials (Table 5.2). PCR analysis again confirmed the presence of TYDV in all symptomatic plants (Table 5.2).

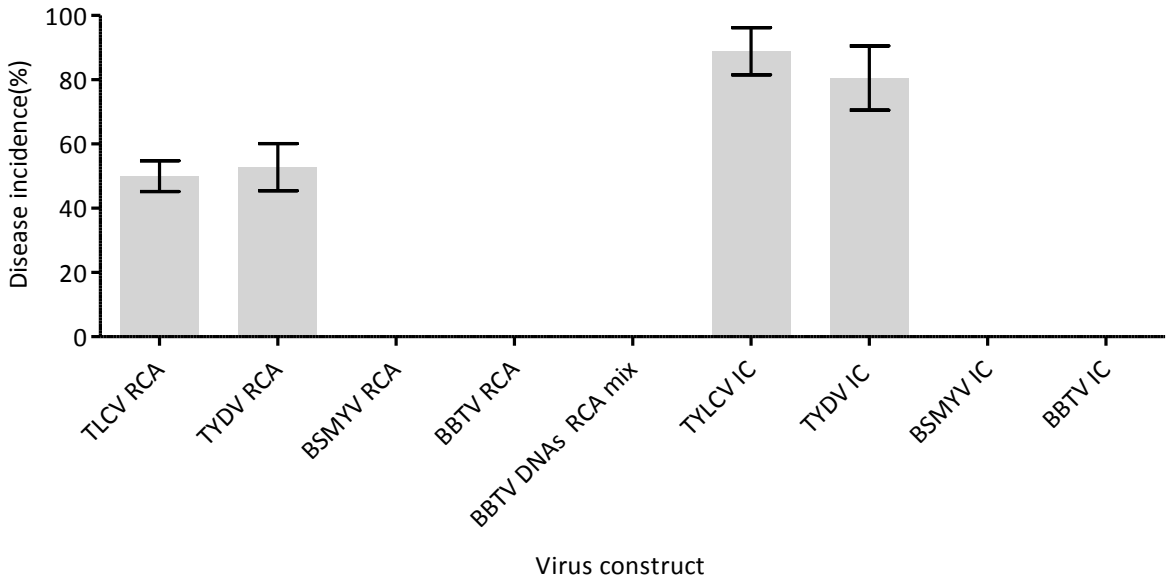


Figure 5.10: Bar graph representing the average disease incidence in tobacco plants at 12 weeks post-inoculation and banana plants at 12 weeks post-inoculation bombarded with different virus constructs in three separate trials. The error bars represent standard deviation (SD) from the mean of disease incidence. IC: infectious clone/construct; RCA: Rolling-circle amplification generated constructs.

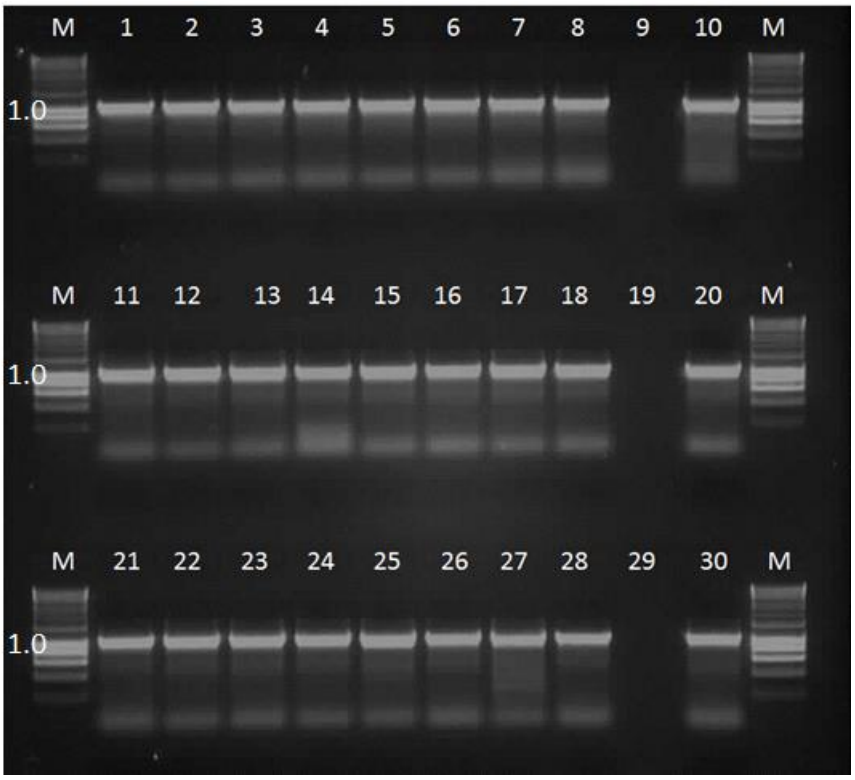


Figure 5.11: PCR screening for TYLCV presence in tobacco plants mechanically inoculated with the TYLCV infectious clone at 12 weeks post-inoculation. Lanes 1-8, 11-18 and 21-28 represent 24 tobacco plants inoculated with the infectious clone, lanes 9, 19 and 29 are non-inoculated control plants and lanes 10, 20and 30 are positive controls (plasmid DNA). The expected size of the PCR product is approximately 1 kbp.

When banana plants were bombarded with either the BSMYV infectious clone, or plasmids containing cloned BBTV DNAs, no plants developed symptoms 12 weeks post-inoculation. Furthermore, none of the inoculated plants were PCR positive when tested for BBTV or BSMYV (Table 5.2). Despite repeating the experiments three times, no banana plants were found to be infected with BBTV or BSMYV by mechanical inoculation.

5.3.5 Agrobacterium-mediated inoculation of banana using cloned BBTV DNAs

Due to the inability to infect banana plants with either BBTV or BSMYV using the Helios gene gun, attempts were made using agro-inoculation of cloned genomic components. The BBTV monomeric constructs in pOPT-EBX (5.2.3) were delivered into banana plantlets by injecting pseudostems with recombinant *Agrobacterium*. As a control for agro-inoculation experiments, banana plants were injected with *Agrobacterium* harbouring the BSMYV infectious clone. Inoculated plants were maintained in a controlled environment growth facility and monitored weekly for symptom development. Banana plants were inoculated on three separate occasions and in each experiment 14 plants were inoculated. At approximately 12 weeks post-inoculation, plants were screened for BSMYV and BBTV using PCR (Fig 5.12).

No banana plants injected with *Agrobacterium* containing the cloned BBTV components developed disease symptoms 12 weeks post-inoculation, and no plants tested positive for BBTV by PCR. In contrast, all plants which were agro-inoculated using the BSMYV infectious clone developed typical symptoms and were PCR positive (Fig 5.13).

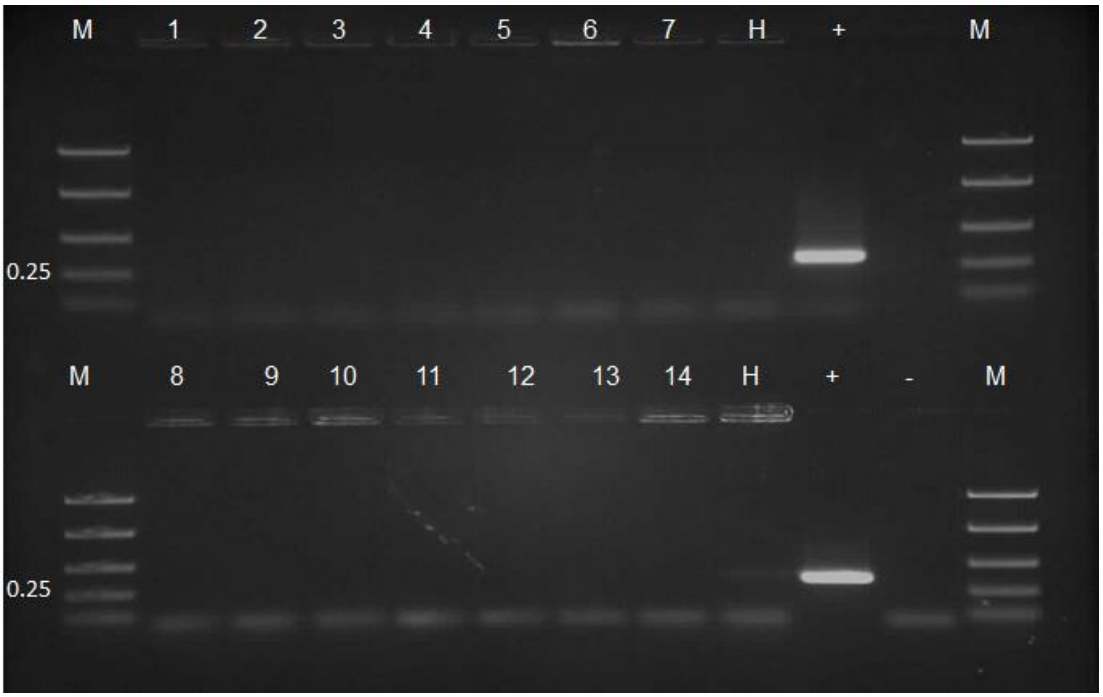


Figure 5.12: PCR screening for BBTV in banana plants agro-inoculated with BBTV DNA monomers in plasmid vector at 12 weeks post-inoculation. Lanes 1-14 represent inoculated plants, H is the non-inoculated control, + is plasmid DNA control and M M-is the Bioline EasyLadder. The expected size of the PCR product is 340 bp.

Figure 5.13: Photograph of banana plants agro-inoculated with BBTV constructs and BSMYV infectious clone at 12 weeks post-inoculation. Plants 1 and 2 were agro-inoculated with BBTV DNA monomers and do not have symptoms, whereas plant 3 was agro-inoculated with the BSMYV infectious clone and has typical symptoms of BSMYV infection.

5.4 Discussion

This chapter focused on developing a method for the mechanical inoculation of bananas with BBTV. To achieve this a range of viral template nucleic acids were tested, including naked RCA-amplified BBTV DNAs or binary vector clones of each virus DNA component in the form of a monomer or dimer and two different inoculation methods were trialled, namely the Helios Gene Gun system for delivery of purified DNAs, or agro-inoculation of binary vectors containing cloned DNA components.

Initially, bombardment conditions for the Helios gene gun system were optimised for banana by varying acceleration pressures (200, 250, 300, 350 and 400 psi) and distances (0, 0.5 and 1 cm) from the leaf tissue surface and monitoring reporter gene expression from a binary vector containing a GUS reporter enzyme. Transient GUS expression was evaluated using histochemical substrate and counting the average number of the blue spots observed on plant tissues three days post-bombardment. The highest average transient GUS expression was observed using a pressure of 250 and 300 psi when banana leaf or pseudostem tissues were bombarded, respectively. The average GUS expression was significantly lower when a pressure of 350 or 400 psi was used, probably due to the severe damage to the tissues at these pressures. Decreasing the distance to 0 cm resulted in severe damage to leaf tissue at all acceleration pressures greater than 250 psi, while a distance of 1 cm led to reduced damage to the leaf surface but few spots of GUS expression. Therefore, the optimum conditions for bombardment of banana were determined to be an acceleration pressure of 250 psi and a distance of 0.5 cm from the plant surface and these conditions were subsequently used for all banana bombardment experiments.

As controls for the inoculation of banana, tobacco plants were bombarded using either TLCV or TYDV prepared using either RCA or as plasmid constructs with cloned copies of the virus genomes. Bombardment was carried out at 200 psi and assessment, both visual and by PCR, confirmed a rate of infection of 75-100%. This result confirmed the efficacy of the Helios Gene Gun for mechanical inoculation of plants and is consistent with a previous study by Lapidot *et al.* (2007) where bombardment of plasmid DNA containing a dimeric form of TYLCV resulted in between 50 and 100% infection in *Datura stramonium* and 37% infection in tomato.

When the Helios Gene Gun was used to inoculate bananas using DNA prepared by RCA, or using plasmid DNA containing cloned copies of the BBTV or BSMYV genomes, none of the bombarded plants became symptomatic or tested positive for the respective virus. For initiation of virus infection, it is likely that at least one copy of each of the integral genomic components of a multipartite virus be delivered into a single amenable cell type at the same time (Grigoras *et al.*, 2009; Iranzo & Manrubia, 2012; Sicard *et al.*, 2013). Perhaps this minimum requirement was not achieved because the DNA was not delivered into an appropriate cell type that can support the BBTV life cycle or perhaps all six BBTV DNA components could not be evenly accommodated on the surface area of a single gold particle. Alternatively, whole banana plants may simply be recalcitrant to infection with cloned virus genomes delivered by particle bombardment. This is emphasized by the fact that a BSMYV infectious clone was capable of initiating an infection in banana using agro-inoculation but not with micro-projectile bombardment. Although GUS expression was observed in bananas following bombardment of plasmid DNA, it may be necessary to widen the range of acceleration pressures and shooting distances assessed for virus inoculation, since particle acceleration directly influences both the depth of penetration and the distribution of particles on the shot surfaces (Mahdavi *et al.*, 2014) all of which directly determine entry of the gold particles into a cell. In this study two key particle bombardment parameters (ie. acceleration pressure and shooting distance) were assessed. Future work may consider testing other variables such as the number of shots, gold particle size, or varying DNA concentration and amount of gold particles; factors which have been shown to influence the success of biolistic-delivery of DNA into banana (Mahdavi *et al.*, 2014).

Agro-inoculation of plants with infectious clones has been successfully used for many plant viruses (Ferreira *et al.*, 2008; Grigoras *et al.*, 2009; Sicard *et al.*, 2013). To investigate if cloned monomers of the six integral components of BBTV could initiate an infection in bananas they were transformed into *Agrobacterium* and inoculated by stem injection. Despite the success of this method with BSMYV, no BBTV infection resulted in banana plants agro-inoculated with BBTV. As with particle bombardment, it is possible that this method was also not capable of delivering the required copy of each BBTV DNA component into a single appropriate cell type. The set of genome length constructs used in this study were

prepared by partial cleavage with *Stu*I, *Spe*I, *Sca*I, *Kpn*I, *Mfe*I and *Pac*I before cloning into a plasmid backbone. It was noted that some of these enzymes, notedly *Stu*I, *Kpn*I and *Mfe*I, were cutting their respective DNAs within the open reading frame and consequently this may have hindered the expression of a functional gene or protein essential for virus infection. In previous studies, dimeric constructs of FBNSV were agro-inoculated into *Vicia faba* plants and shown to reproduce typical symptoms ((Grigoras *et al.* (2009); Sicard *et al.* (2013)).

As an alternative to cloning of digested BBTV genomic DNAs, a PCR-based approach was used to amplify full-length components of each BBTV DNA. Outward-facing primers were used for priming amplification and PCR products were cloned into a binary vector for agro-inoculation. As before, these constructs did not induce infection following repeated agro-inoculation of banana plants. Sequencing of PCR-amplified sequenced showed that although functional ORF sequences were present a number of nucleotide changes were identified which may have interfered with expression or otherwise affected the integrity of the constructs.

Future efforts to generate a BBTV infectious clone would benefit from using clones with 100% sequence similarity to the original isolate, or at least with minimal nucleotide variation from published sequences. This could be achieved through chemical synthesis of published sequences to eliminate nucleotide mismatches/changes and either monomeric or dimeric forms could be prepared this way and delivered using agro-inoculation.

CHAPTER 6

INVESTIGATION INTO THE EFFECT OF BANANA GENOTYPE ON BBTV SUSCEPTIBILITY UNDER GLASSHOUSE CONDITIONS

6.1 Introduction

Despite its rich genetic diversity, the existence of bananas is threatened by a range of important diseases. Today, more than 90% of dessert bananas for commercial export are generated from a single cultivar called Cavendish (AAA). The reliance on a single cultivar limits genetic variation in production systems and predisposes a crop to disease pandemics. It is believed that many wild bananas contain genes that confer resistances to many biotic and abiotic stresses (Wang *et al.*, 2007). The challenge lies with exploiting these genes in commercial banana varieties, most of which are highly susceptible to many pathogens and insect pests (Lu *et al.*, 2011). One of the major constraints to banana production systems throughout the world (with the exception of Latin America) is *Banana bunchy top virus* (BBTV). Although no bananas with immunity to BBTV have been reported to date, there appear to be differences in the susceptibility of cultivars depending on genotype.

Two field studies have assessed the response of a diverse collection of banana genotypes to BBTV infection (Niyongere *et al.*, 2011; Niyongere *et al.*, 2013). In the 2011 study, no bunchy top symptoms were observed in *Musa balbisiana* type Tani (BB), or in 'Kayinja' (ABB), 'FHIA-03' (AABB), 'Prata' (AAB), 'Gisandugu' (ABB), 'Pisang Awak' (ABB), 'Saba' (ABB) and 'Highgate' (AAA, Gros Michel subgroup) twenty-eight months after planting. When plants were tested for virus presence by PCR, BBTV was only detected in Pisang Awak, Saba and Highgate, with the latter shown to be a symptomless host (Niyongere *et al.*, 2011). The detection of BBTV in Highgate (Gros Michel subgroup) was unexpected as disease resistance attributes have generally been associated with the B-genome (Niyongere *et al.*, 2011). The 2013 study subsequently confirmed that cultivars containing some B-genome component displayed lower bunchy top incidence (Niyongere *et al.*, 2013).

In addition to data on disease incidence, the response of plant cultivars to virus infection can also be characterised based on the levels of virus DNA or RNA accumulation in respective cultivars or genotypes (Šíp *et al.*, 2006). Multipartite viruses comprise more than

one genomic component and these have been shown to accumulate with varying frequencies dependent on the host plant genotype. For example, the different viral genomic DNA components of *Faba bean necrotic stunt virus* (FBNSV; family *Nanoviridae*) were shown to accumulate at different levels in Faba bean plants (Sicard *et al.*, 2013). Based on qPCR studies the ratio of each genomic component can be determined leading to the establishment of a 'genome formula' or the relative proportion of each of the genomic segments. Such relative proportions have been shown to be consistent across cultivars/genotypes, suggesting that within infected plants there is a tight regulation of the ratio of each viral DNA component (Sicard *et al.*, 2013).

BBTV is a multipartite virus in the family *Nanoviridae*. The level of BBTV DNA accumulation and the ratio of each BBTV DNA component within infected plants have not been studied. This chapter aimed to characterize the response of a diverse collection of banana genotypes to BBTV infection under glasshouse conditions and determine the amount and ratios of the each BBTV component in susceptible bananas of different genetic backgrounds.

The specific objectives of this chapter were to:

1. Evaluate the response of different banana genotypes to BBTV infection under glasshouse conditions
2. Quantify BBTV DNA accumulation in different banana genotypes by real time PCR and investigate changes in virus DNA accumulation over time
3. Determine BBTV DNA component ratios in infected plants
4. Establish a BBTV genome formula

6.2 Methods and materials

6.2.1 Banana genotypes

Tissue cultured plants of Cavendish subgroup cv. Dwarf Cavendish (AAA) and Lady Finger (AAB) were available from the CTCB. Nine additional *Musa* genotypes (both wild and cultivated) were provided by the Queensland Department of Agriculture, Fisheries and Forestry (DAFF) as tissue cultured plantlets including Gros Michel (AAA), *M. acuminata* ssp. *zebrina* (AA), Pisang Bangkahulu (AA), Akondro Mainty (AA), Khae Phrae (AA), Ney Poovan (AB), Pisang Gajih Merah (ABB), Saba (ABB) and Butuhan (*M. balbisiana*, BB).

6.2.2: Tissue culture multiplication & glasshouse acclimatisation

Banana plantlets were cultured on multiplication media, with subculturing at four-weekly intervals, as described in section 4.2.1 until sufficient replicates were generated for glasshouse trials. Plantlets were then transferred to rooting media (section 3.2.7) for a further 8-12 weeks until sufficient roots had developed, prior to transport to the glasshouse for acclimatisation. Plants were acclimatised as described in section 4.2.2.

6.2.3: BBTV inoculation, monitoring for symptoms and PCR detection of BBTV

Plants were inoculated using a local south-east Queensland BBTV (South Pacific subgroup) isolate as described in section 4.2.3 and monitored for the development of symptoms as described in section 4.2.4. Disease incidence was recorded weekly based on a visual assessment of plants for typical bunchy top symptoms and leaf sampling for PCR was carried out fortnightly throughout the experiment, commencing from five weeks post-inoculation. Total nucleic acid was isolated as described in section 2.5.1, diluted to a concentration of 10 ng/µL and subjected to end-point PCR screening for the presence of BBTV as described in section 2.4.2. Primers used were designed to amplify a 647 bp fragment of BBTV DNA-C.

6.2.4 Absolute quantification by real-time (rt) PCR

rtPCR was carried out as described in sections 2.8.6 and 4.2.6 using the total nucleic acid extracts diluted to 10 ng/µL. Primers (Table 6.1) were designed based on published BBTV South Pacific subgroup sequences to amplify a region within the ORF of each of the six BBTV DNAs. Each rtPCR amplicon was cloned into pGemT-Easy and sequenced (as per section 3.2.2) and purified plasmid DNA was then used to prepare standard curves for absolute quantification of each BBTV DNA component as described in section 4.2.6. To eliminate the effect of background amplification in rtPCR, all measurements were corrected by subtracting the average copy number calculated in non-inoculated plants of each genotype from the average copy number of each inoculated plant of each genotype. For each genotype, three symptomatic plants were selected for analysis. Calculated virus DNA levels from three plants were subjected to statistical analysis using ANOVA to identify results which were significantly different.

Table 6.1: Primer sequences for rtPCR

Target BBTV component	Virion sense primer (5' to 3')	Complementary sense primer (5' to 3')	Amplicon size (bp)
DNA-R	CAGGAGGAAAATCATTGGAT	CTATCTTCAAAACGGGTTCA	162
DNA-U3	GGTAACCGGTCAACATTATT	CCTTGACAGGATTAGGGTAT	123
DNA-S	TATGGCAGCAAGGCGGCAAC	TCCGGGCTTCACCTTGCACA	165
DNA-M	GCTCGTGAGGTGTTTGGTAG	GGTATGACAGCCGGTTCTTG	141
DNA-C	CCATGTGTGGTAATTTGTTG	GATGCCATGATAACATTCCT	170
DNA-N	GATATCATCGGATTCAGCCG	CCGTTAAACACAGCTTCAAT	106

6.3 Results

The 11 banana genotypes were multiplied, acclimatised in the glasshouse and inoculated with viruliferous aphids in two groups. Dwarf Cavendish plants were included as a susceptible control in both groups to monitor inoculation rates between groups. Non-inoculated control plants for each genotype were maintained in a separate glasshouse compartment. Prior to inoculation, all plants were tested for BBTV by PCR. No amplicons were generated from extracts derived from any of the test plants with the exception of the known BBTV-infected positive control sample (results not shown). Following inoculation with viruliferous aphids, symptom development and disease incidence were monitored visually for up to eight months. Banana leaf samples were collected fortnightly from week 5 post-inoculation for PCR analyses. An end-point PCR assay was used to confirm the presence of BBTV in plants, followed by absolute quantification of all six BBTV DNAs using rtPCR.

6.3.1 Bunchy top incidence

In the first inoculation experiment, 7-10 plants of each of four banana genotypes (Dwarf Cavendish (AAA), Gros Michel (AAA), Pisang Gajih Merah (ABB) and Saba (ABB)) were exposed to viruliferous aphids (Table 6.2). Symptoms of bunchy top were first observed in some Dwarf Cavendish plants three weeks post-inoculation, with all plants showing symptoms by week 12. In contrast, no symptoms were observed on any of the nine Gros Michel, seven Pisang Gajih Merah and seven Saba plants at 12 weeks post-inoculation. To confirm the presence/absence of BBTV in the plants, total nucleic acid was extracted from each plant at 12 weeks post-inoculation and used as a template in a PCR. A PCR product of the expected size of 647 bp was amplified from extracts from all 10 symptomatic Dwarf Cavendish plants (Table 6.2), while all of the symptomless Gros Michel, Pisang Gajih Merah and Saba plants tested negative, thus confirming the visual diagnosis.

The experiment was continued for an additional five months to allow further monitoring for symptom development in the test plants. Leaf sampling was continued fortnightly to enable virus testing by PCR. When samples collected from the seven Pisang Gajih Merah were tested at week 18, one plant tested positive for BBTV by PCR despite no obvious bunchy top symptoms. At 28 weeks post-inoculation, symptoms were observed on this plant. Two additional symptomless plants tested PCR positive at week 20, and these two plants

Figure 6.2: Effect of banana genotype on susceptibility to BBTV under glasshouse conditions

Inoculation group	Cultivar name	Genotype	No. of plants inoculated	No. of plants with symptoms	No. of PCR positive plants	BBTV incidence (%)
1	Dwarf Cavendish	AAA	10	10	10	100
	Gros Michel	AAA	9	0	0	0
	Pisang Gajih Merah	ABB	7	0	3	43 [a]
	Saba	ABB	7	0	0	0
2	Dwarf Cavendish	AAA	10	10	10	100
	Gros Michel	AAA	10	1	1	10
	M. acuminata ssp. *zebrina*	AA	10	8	8	80
	Pisang Bangkahulu	AA	10	6	6	60
	Akondro Mainty	AA	10	6	6	60
	Khae Phrae	AA	10	0	0	0
	Ney Poovan[b]	AB	10	0	1	10
	Lady Finger	AAB	10	8	8	80
	Butuhan (*M. balbisiana*)	BB	8	0	0	0

[a] No plants had developed symptoms at 12 weeks post-inoculation, however by 28 weeks post-inoculation three plants had developed symptoms and were PCR positive

[b] Although no plants of Ney Poovan developed symptoms, one plant tested positive for BBTV by PCR

developed symptoms at 32 weeks post-inoculation, giving a final disease incidence of 43% in this cultivar (Table 6.2).

None of the Saba and Gros Michel plants developed symptoms during the eight month period and none tested positive for BBTV by PCR when tested at various time points. At the completion of the 8 month observation period, all plants were tested for BBTV by PCR. The results of the PCR testing were again consistent with the visual assessments, with all inoculated and symptomatic Dwarf Cavendish and three out of seven symptomatic Pisang Gajih Merah plants testing positive for BBTV. Further, all of the inoculated Saba and Gros Michel plants tested negative for BBTV, along with the non-inoculated plants.

The second inoculation experiment included Dwarf Cavendish (AAA) controls, Gros Michel (AAA), four additional A-only (diploid-AA) genotypes, namely *M. acuminata* ssp. *zebrina*, Pisang Bankahulu, Akondro Mainty and Khae Phrae and three genotypes with some B-genome component, namely Ney Poovan (AB), Lady Finger (AAB) and Butuhan (BB). At 12 weeks post-inoculation, all 10 Dwarf Cavendish plants showed typical disease symptoms with the initial symptom emergence recorded at week 3 post inoculations. For Gros Michel plants, symptom emergence was delayed with only 1/10 plants showing symptoms in form of narrow leaves at week 12. Of the AA cultivars, 8/10 *M. acuminata* ssp. *zebrina*, 6/10 Pisang Bankahulu and 6/10 Akondro Mainty plants showed symptoms while all of the 10 Khae Phrae plants remained symptomless. Of the 10 inoculated Lady Finger (AAB) plants, eight showed symptoms while none of the eight Butuhan (BB) plants showed bunchy top symptoms (Table 6.2).

Under normal growing conditions, *Musa acuminata* ssp. *zebrina* exhibits a distinct purple mosaic pattern on the leaf lamina. However, BBTV infection in this genotype led to a general reduction of the purple pigment on newly formed leaves as well as bright chlorotic margins that progressively spread towards the midrib. Simliarly, Pisang Bankahulu and Akondro Mainty usually have green leaves with scattered patches of purple pigmentation. Upon infection by BBTV, newly formed leaves of both cultivars exhibited chlorotic margins spreading towards the midrib (Fig 6.1). Interestingly, BBTV-infected plants of cultivar Akondro Mainty developed the typical dark green streak symptoms along the midrib on the

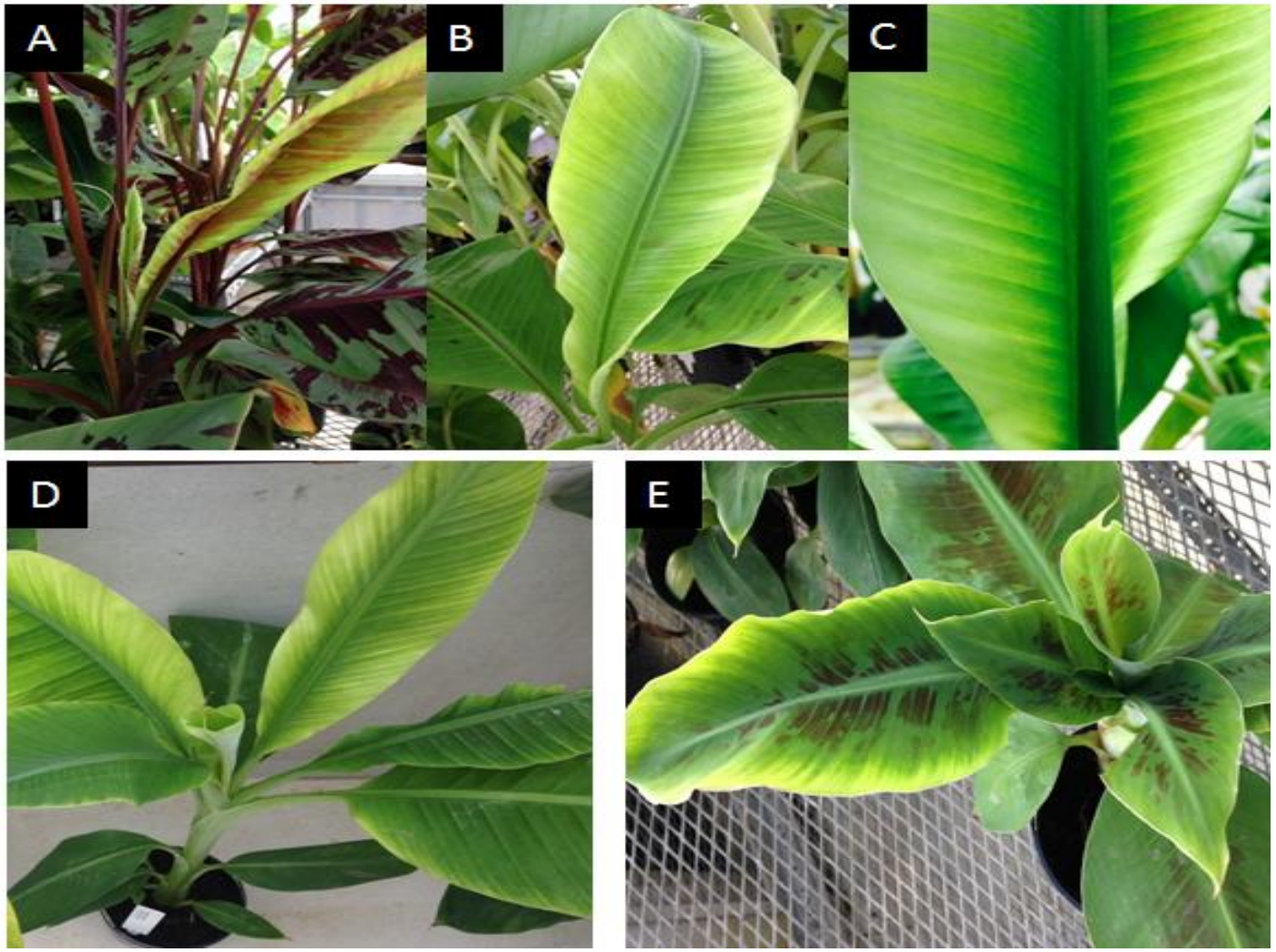


Figure 6.1: Bunchy top symptoms in different banana genotypes; A) *M. acuminata* ssp. *zebrina*; B) Pisang Bankahulu; C) Akondro Mainty; D) Lady Finger and E) Dwarf Cavendish.

back side of the leaf, however, at later stages of disease progression the leaf margins dried, followed by necrosis and death of the entire leaf.

When wild-type Dwarf Cavendish control plants were assayed for BBTV by PCR at 12 weeks post-inoculation, the results were consistent with the visual diagnosis based on symptoms (Fig 6.2). This was also the case in all of the other genotypes tested with a single exception of one Ney Poovan plant which did not show symptoms but tested positive for BBTV.

Plants of Ney Poovan, Butuhan and Khae Phrae were maintained in the glasshouse for a further three months to monitor for delayed symptom development. None of the plants developed symptoms during this time and, with the exception of the single Ney Poovan plants which tested positive for BBTV at 12 weeks post-inoculation, all plants tested negative for the virus by PCR.

6.3.2 rtPCR

To determine the absolute level of BBTV accumulation in each cultivar, rtPCR was carried out. Prior to quantification of the amount of BBTV in selected test plants, plasmid controls for each of the six BBTV DNA genome components were prepared from which standard curves were constructed to determine the absolute quantity of BBTV DNAs present in selected replicates. A ten-fold serial dilution of each plasmid containing the target amplicon was quantified in triplicate and a linear regression curve was generated by plotting the mean cycle threshold (Ct) values against the log of the copy number of the target amplicons. Standard curves with coefficients of correlation (R^2) of between 0.99-1.0 were generated with amplification efficiencies ranging from 96-100%. The plasmid standards were then used for absolute quantification of BBTV DNAs.

6.3.2.1 BBTV DNA accumulation at a given sampling point in different banana genotypes

Of the 11 genotypes included in the inoculation experiments, plants from four banana cultivars with distinct genotypes (Dwarf Cavendish (AAA), Lady Finger (AAB), *Musa acuminata* ssp. *zebrina* (AA) and Pisang Gajih Merah (ABB)) were selected for subsequent rtPCR analysis. For each genotype, three plants were randomly selected from those which had tested positive for BBTV using conventional PCR. For cultivars Dwarf Cavendish, Lady

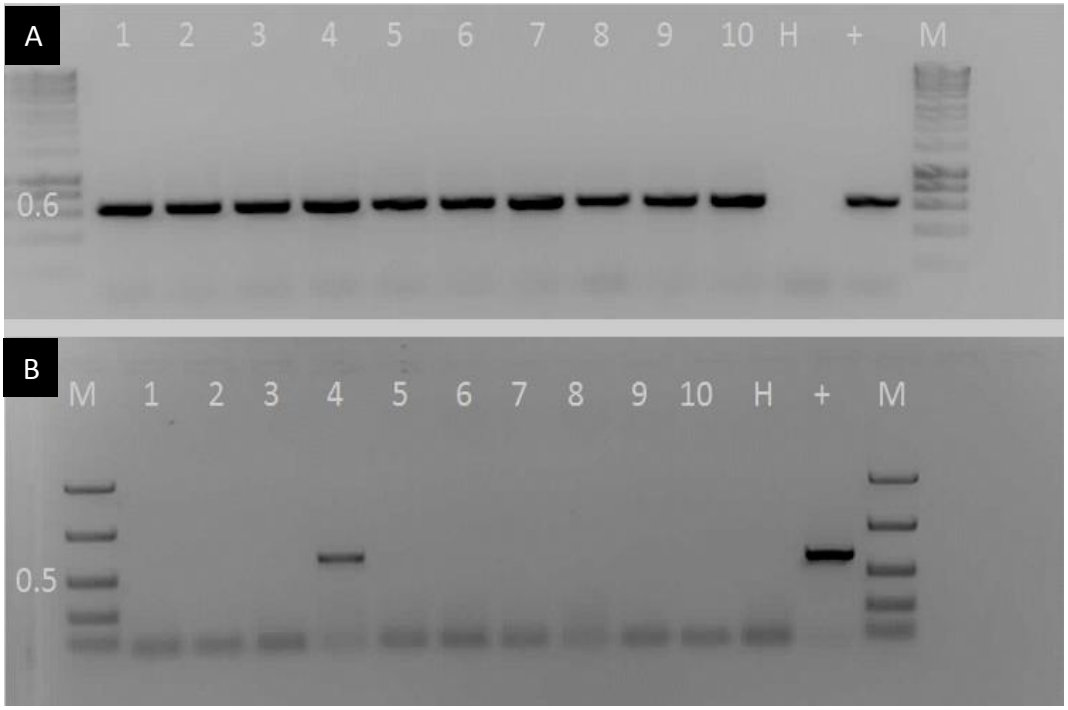


Figure 6.2: PCR screening for BBTV in inoculation experiment 2. The results of testing A) Dwarf Cavendish plants and B) Ney Poovan plants are shown with replicates for each cultivar labelled 1-10; lane 'M' is the molecular weight marker (Bioline HyperLadder 1 in A or EasyLadder 1 in B); lane 'H' is the non-inoculated control plant and lane '+' is the PCR positive control.

Finger and *Musa acuminata* ssp. *zebrina*, leaf samples for analysis were initially collected at nine weeks post-inoculation, a time point where at least three replicates of each cultivar had developed bunchy top symptoms. For plants of cultivar Pisang Gajih Merah, leaf samples were not taken until week 20 since it was only at this time point that the three symptomless plants of this cultivar were shown to be BBTV-infected. Total genomic DNA was extracted from the leaf samples and was diluted to a concentration of 10 ng/µL for subsequent qPCR analysis.

When qPCR results for each BBTV DNA from the three replicates of cultivar Dwarf Cavendish were analysed, DNA-N was found to be the most abundant virus DNA (Fig 6.3 & 6.4). The level of DNA-N in infected Dwarf Cavendish plants was eight-fold higher than the next most abundant virus DNA, DNA-U3, which was more than two-fold higher than the level of DNA-R. DNA-M, -S and –C were the least abundant BBTV DNAs in Dwarf Cavendish plants, with DNA-M approximately five-fold higher than DNA-S, while the level of DNA-S and –C was not significantly different ($P<0.0001$) (Fig 6.3 & 6.4). The level of DNA-N was 22-fold higher than DNA-R, 111-fold higher than DNA-M and 556-fold higher than DNA-S and –C.

Similarly, with cultivar Pisang Gajih Merah, DNA-N was again shown to be the most abundant virus DNA, followed by DNA-U3 and DNA-R (Fig 6.3 & 6.4). The level of DNA-N in infected Pisang Gajih Merah plants was more than seven-fold higher than DNA-U3 which had more than two-fold higher DNA accumulation compared to DNA-R. As in Dwarf Cavendish, DNA-M, -S and –C were present in lowest amounts, with DNA-M approximately four-fold higher than DNA-S and –C, which were again not significantly different ($P<0.0001$) from each other. The level of DNA-N in plants of cultivar Pisang Gajih Merah was more than 16-fold higher than DNA-R, more than 71-fold higher than DNA-M and 286-fold higher than DNA-S and –C.

DNA-N was again shown to be the most abundant virus DNA in the three plants of cultivar Lady Finger, followed by DNA-U3 (Fig 6.3 & 6.4). The level of DNA-N in infected Lady Finger plants was more than 13-fold higher than DNA-U3. Although DNA-R was shown to be the next most abundant BBTV DNA, there was no significant difference between the levels of DNA-U3 and DNA-R in BBTV-infected Lady Finger plants. Quantification of BBTV DNA-S, -M

and –C revealed these were again the least abundant BBTV DNAs and, as previously DNA-M was slightly higher with approximately two-fold greater DNA accumulation compared to DNA-S and –C, while DNA-S and –C were not significantly different ($P<0.0001$). The level of DNA-N was 21-fold higher than DNA-R, 132—fold higher than DNA-M and 345-fold higher than DNA-S and –C.

With *Musa acuminata* ssp. *zebrina*, DNA-N was again found to be the most abundant virus DNA followed by DNA-U3, DNA-R, DNA-M and then DNA-S/-C (Fig 6.3 & 6.4). The level of DNA-N in infected *Musa acuminata* ssp. *zebrina* plants was more than eight-fold higher than DNA-U3, 45-fold higher than DNA-R, 173-fold higher than DNA-M and more than 188-fold higher than DNA-C. In contrast to the other three cultivars tested, there was a significant difference between the level of DNA-S and –C, with DNA-C 3-fold higher than DNA-S.

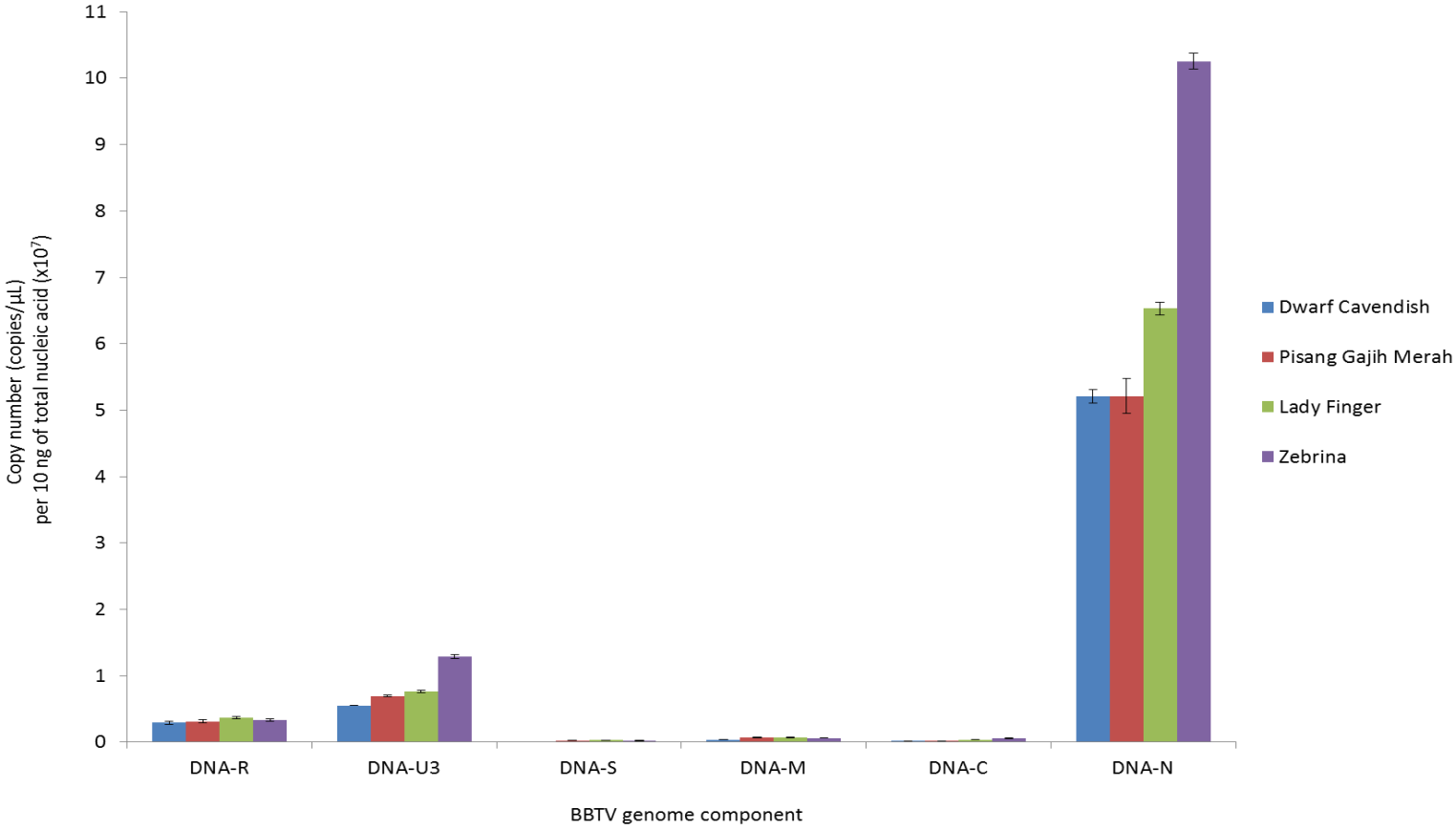


Figure 6.3: Absolute quantification of each of the six BBTV DNAs in BBTV-infected plants from four cultivars, namely Dwarf Cavendish (AAA), Pisang Gajih Merah (ABB), Lady Finger (AAB) and *Musa acuminata* ssp. *zebrina* (AA, labelled as 'Zebrina'). Values shown are the average from three plants at each sampling time point, being nine weeks post-inoculation for Dwarf Cavendish, Lady Finger and *Musa acuminata* ssp. zebrina and 20 weeks post-inoculation for Pisang Gajih Merah.

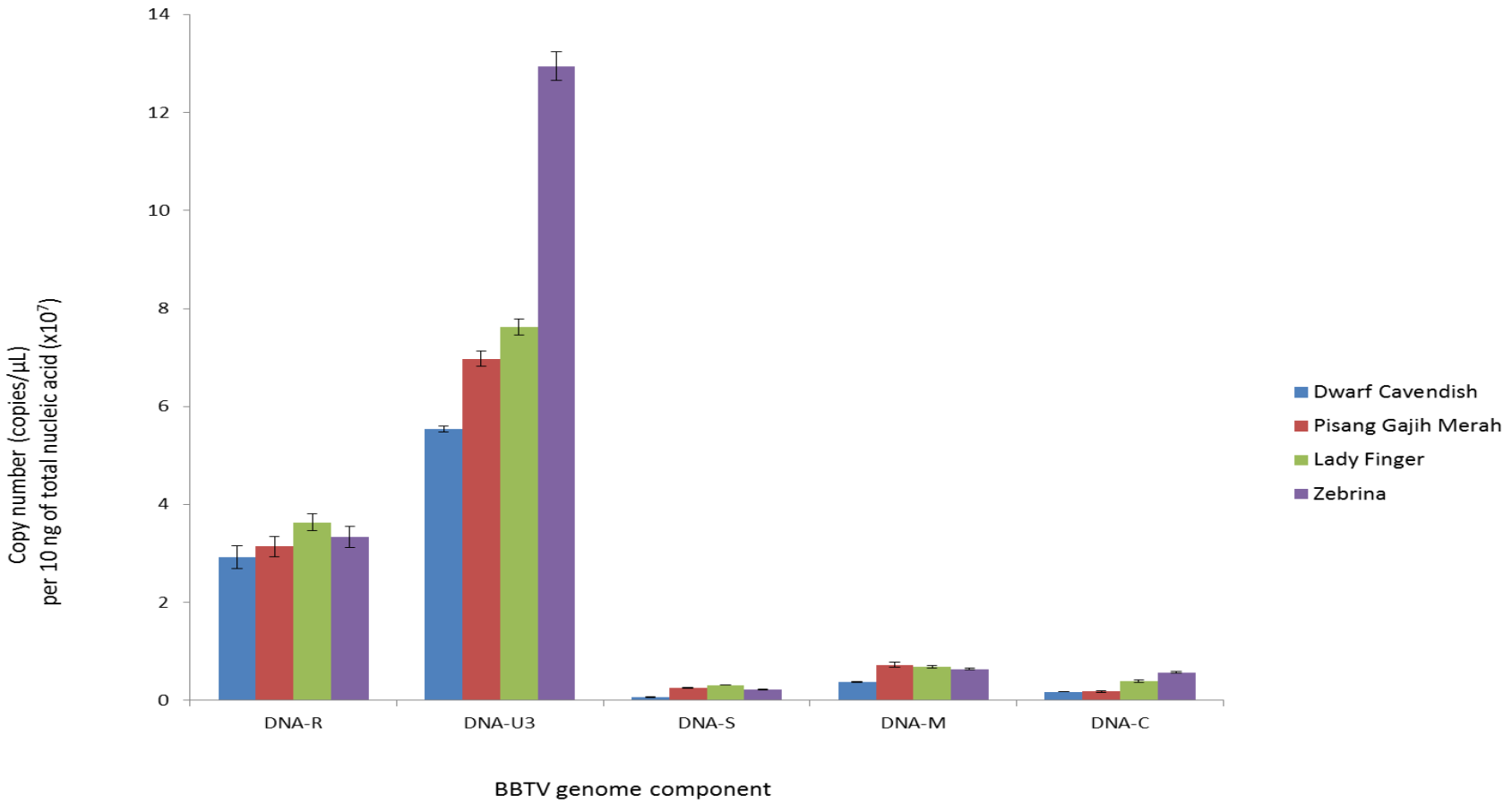


Figure 6.4: Magnified version of the qPCR results presented in Figure 6.3 excluding those for DNA-N. Values shown are the average from three plants at each sampling time point, being nine weeks post-inoculation for Dwarf Cavendish, Lady Finger and *Musa acuminata* ssp. zebrina and 20 weeks post-inoculation for Pisang Gajih Merah.

6.3.2.2 BBTV DNAs accumulation and frequency over time in different banana genotypes

To quantify the amount of each BBTV DNA component in different BBTV-infected banana genotypes over time, samples from newly formed fully unfurled leaves were collected fortnightly over a period of six to eight weeks and were subjected to rtPCR. For cultivars Dwarf Cavendish, Lady Finger and *Musa acuminata* ssp. *zebrina*, samples were collected at weeks 5, 7, 9, and 11, while for Pisang Gajih Merah, samples were collected from weeks 18, 20 and 22 post-inoculation. In all cases, the results reflect the analysis of three individual plants.

When Dwarf Cavendish plants were analysed, the copy number of DNA-R ranged from 7.42 x 10^5 to 4.08 x 10^6 over the 8 week period, with a gradual decline from week 5 to week 9, but increasing significantly between weeks 9 and 11 post-inoculation (Table 6.3, Fig 6.5 & 6.6). In contrast, the copy number of DNA-U3 decreased steadily from 8.66 x 10^6 to 1.76 x 10^6 over the eight week period (an approximately five-fold decrease), with a similar trend observed for DNA-S (9-fold) and –C (31-fold) although significantly fewer copies of these two DNAs were present. With DNA-M, the trend was similar to DNA-R with a decrease in copy number from 2.61 x 10^5 at weeks 5 and 7 to 1.57 x 10^5 at week 9, followed by an increase to 4.22 x 10^5 by week 11. DNA-N, although being present at a significantly higher level than the other DNAs, also followed a similar trend to DNA-R and –M, with the copy number of this virus DNA declining from 5.10 x 10^7 to 1.63 x 10^7 from week 5 to 9, but increasing significantly by week 11.

Table 6.3: Accumulation of BBTV genome components (copies/µL) over time in four different banana genotypes

Dwarf Cavendish	DNA-R	DNA-U3	DNA-S	DNA-M	DNA-C	DNA-N
Week 5	$2.29x10^6$	$8.66x10^6$	$1.09x10^5$	$2.61x10^5$	$4.14x10^5$	$5.10x10^7$
Week 7	$1.64x10^6$	$4.24x10^6$	$4.55x10^4$	$2.76x10^5$	$6.35x10^4$	$3.16x10^7$
Week 9	$7.42x10^5$	$1.96x10^6$	$2.92x10^4$	$1.57x10^5$	$4.25x10^4$	$1.63x10^7$
Week 11	$4.08x10^6$	$1.76x10^6$	$1.16x10^4$	$4.22x10^5$	$1.30x10^4$	$5.74x10^7$
Lady Finger						
Week 5	$4.77x10^5$	$4.14x10^6$	$4.96x10^5$	$1.89x10^5$	$6.35x10^5$	$3.70x10^7$
Week 7	$2.37x10^6$	$8.63x10^6$	$1.40x10^5$	$7.05x10^5$	$2.07x10^5$	$4.62x10^7$
Week 9	$2.73x10^6$	$4.44x10^6$	$1.68x10^5$	$4.51x10^5$	$1.99x10^5$	$5.78x10^7$
Week 11	$5.33x10^6$	$5.68x10^6$	$1.38x10^5$	$7.39x10^5$	$1.52x10^5$	$5.49x10^7$
Musa acuminata* ssp. *zebrina						
Week 5	$2.19x10^6$	$1.08x10^7$	$2.82x10^5$	$2.88x10^5$	$7.59x10^5$	$7.94x10^7$
Week 7	$2.51x10^6$	$1.10x10^7$	$1.22x10^5$	$6.42x10^5$	$2.90x10^5$	$7.69x10^7$
Week 9	$2.34x10^6$	$1.15x10^7$	$2.24x10^5$	$5.83x10^5$	$5.44x10^5$	$1.01x10^8$
Week 11	$2.99x10^6$	$5.56x10^6$	$4.59x10^4$	$4.07x10^5$	$1.25x10^5$	$5.01x10^7$
Pisang Gajih Merah						
Week 18	$3.58x10^6$	$5.39x10^6$	$2.06x10^6$	$7.87x10^5$	$2.05x10^6$	$1.24x10^8$
Week 20	$3.14x10^6$	$6.98x10^6$	$2.53x10^5$	$7.28x10^5$	$1.82x10^5$	$5.21x10^7$
Week 22	$8.87x10^6$	$8.24x10^6$	$1.52x10^5$	$1.37x10^6$	$1.46x10^5$	$5.20x10^7$

BBTV DNAs absolute copy numbers quantified by real time PCR in different banana genotype over time. Since emergence of BBTD symptoms was delayed in Pisang Gajih Merah, the real time PCR was conducted from week 18 post virus inoculation. BBTV DNA-N showed the highest absolute copy numbers across the different banana genotypes.

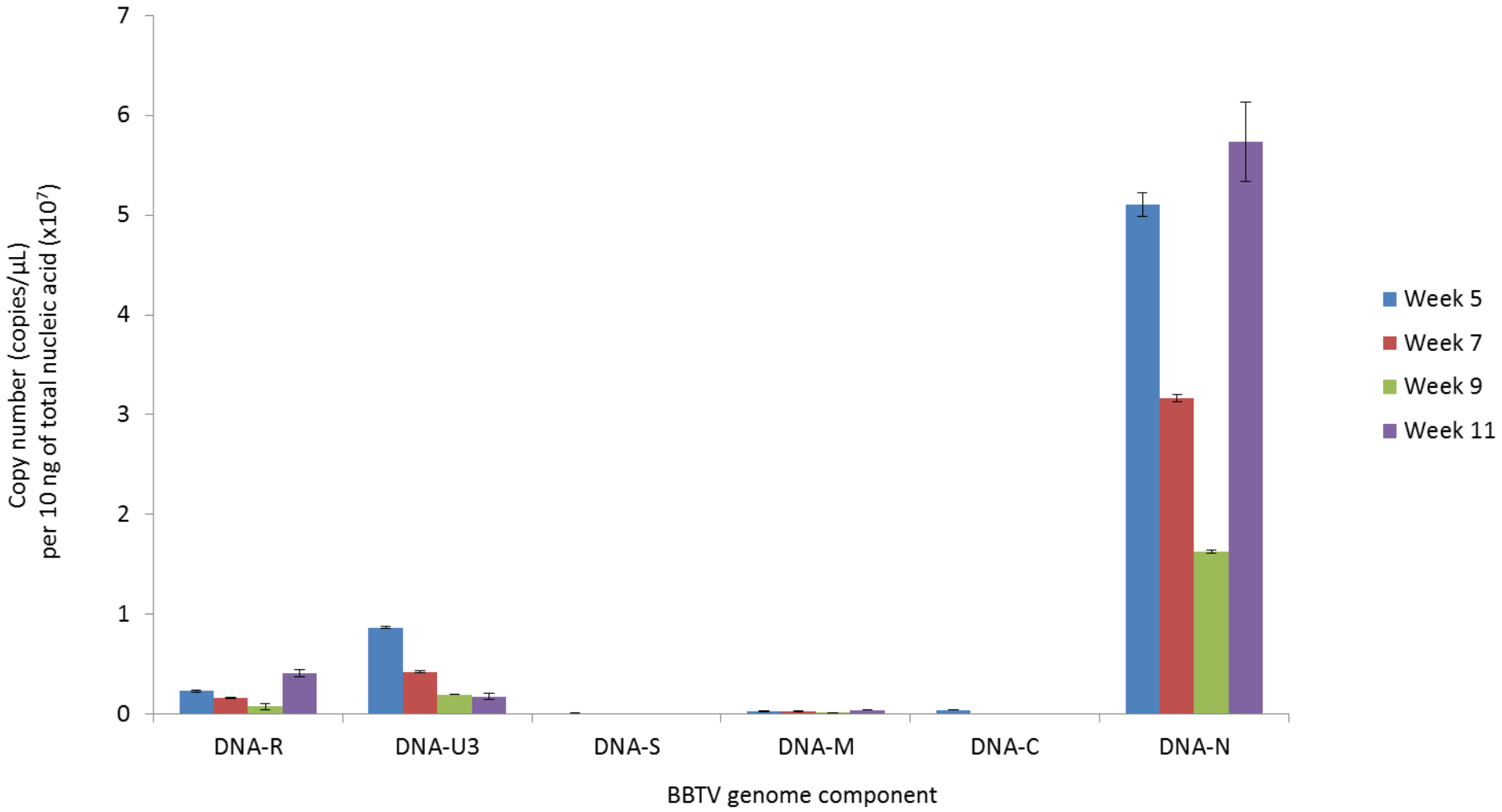


Figure 6.5: Absolute quantification of the six BBTV DNAs in BBTV-infected Dwarf Cavendish plants at sampling intervals of two weeks from week 5 to 11 post-inoculation. Values shown are an average of three plants for each time point. BBTV DNA-N showed the highest absolute copy numbers across the different time points of sampling.

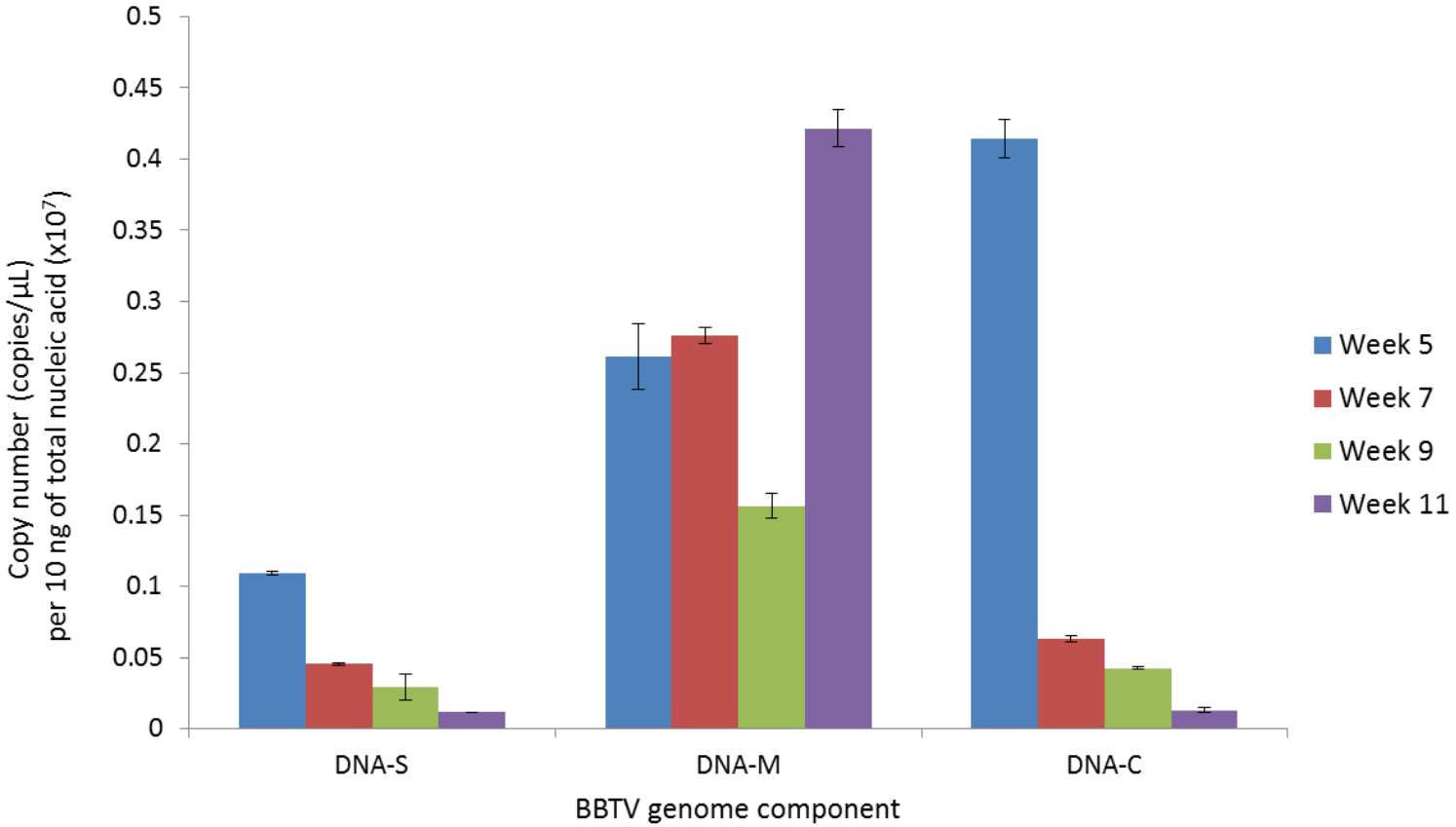


Figure 6.6: Magnified version of the qPCR results presented in Figure 6.5 excluding those for DNA-N, -R and -U3. Values shown are an average of three plants for each time point. Apart from DNA-M at week 11, the absolute copy numbers of the four DNAs decreased from week 7 through to the 11th week.

When Lady Finger plants were analysed by qPCR over the 8 week period, the copy numbers of DNA-R and –N decreased from week 9 to 11 although the level at week 11 was much higher than at week 5 or 7 while those of DNA-S and –C decreased (Table 6.3, Fig 6.7 & 6.8). In contrast, the levels of DNA-U3 and –M varied significantly between sampling times, but no obvious trend was seen. The relative levels of each of the virus DNAs were consistent with the previous findings at nine weeks. In contrast, when *Musa acuminata* ssp. *zebrina* plants were analysed, the copy number of DNA-R was consistently low over the 8 week period while DNA-U3 and –N were not significantly different from week 5 to week 9 but decreased by about 50% at week 11. DNA–M increased from week 5 to 7 but was stable thereafter, while DNA-S and –C were consistently low between sampling times with no obvious trend (Fig 6.9 & 6.10 and Table 6.3). In Pisang Gajih Merah, samples collected at weeks 18 to 22 post-inoculation were analysed as the first BBTV detection in this cultivar occurred at week 18. In this cultivar the level of DNA-R, -U3 and –M generally increased while DNA-S, -C and –N generally decreased (Fig 6.11 & 6.12 and Table 6.3).

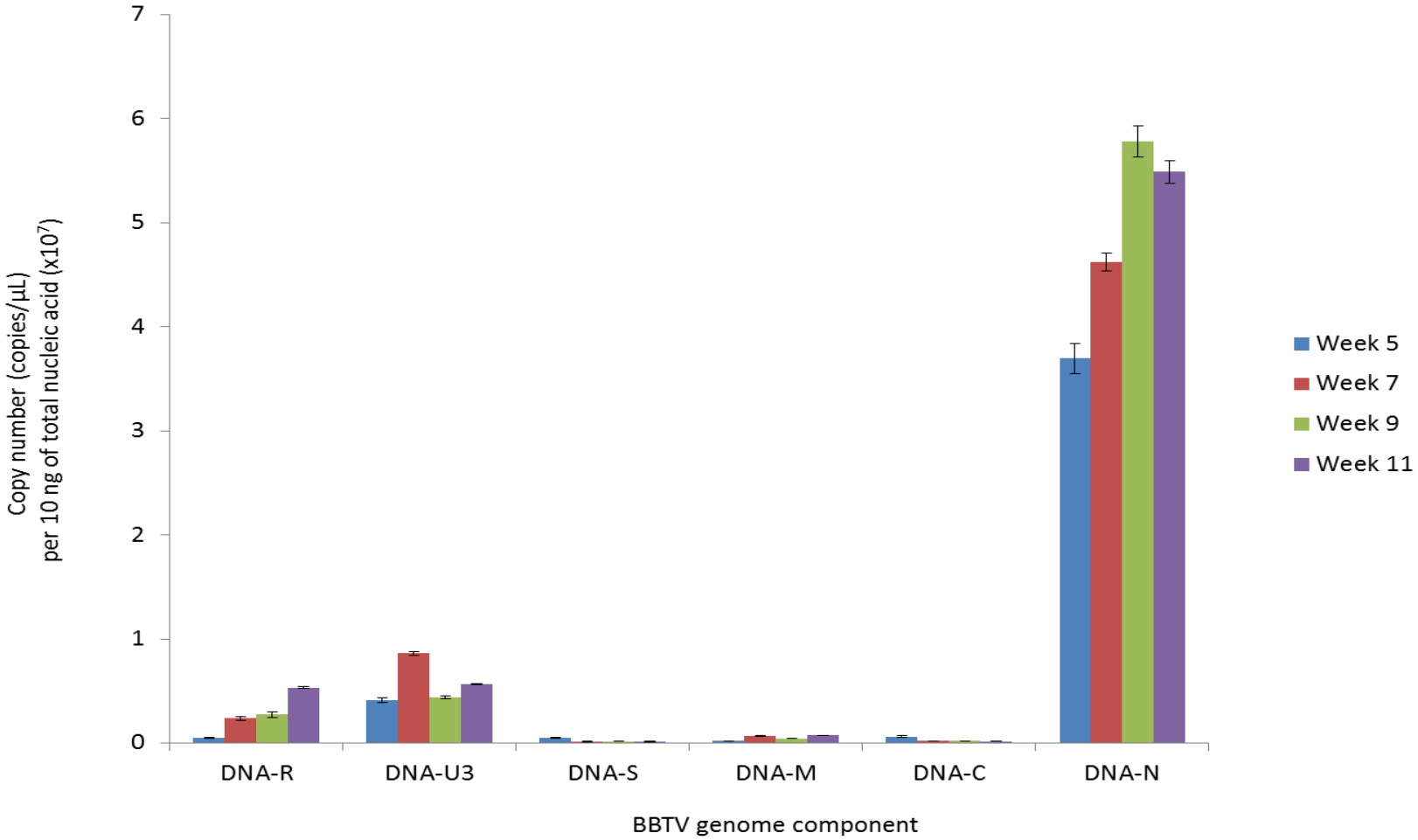


Figure 6.7: Absolute quantification of the six BBTV DNAs in BBTV-infected Lady Finger plants at sampling intervals of two weeks from week 5 to 11 post-inoculation. Values shown are an average of three plants for each time point. BBTV DNA-N displayed the highest absolute copy numbers across the different time points of sampling.

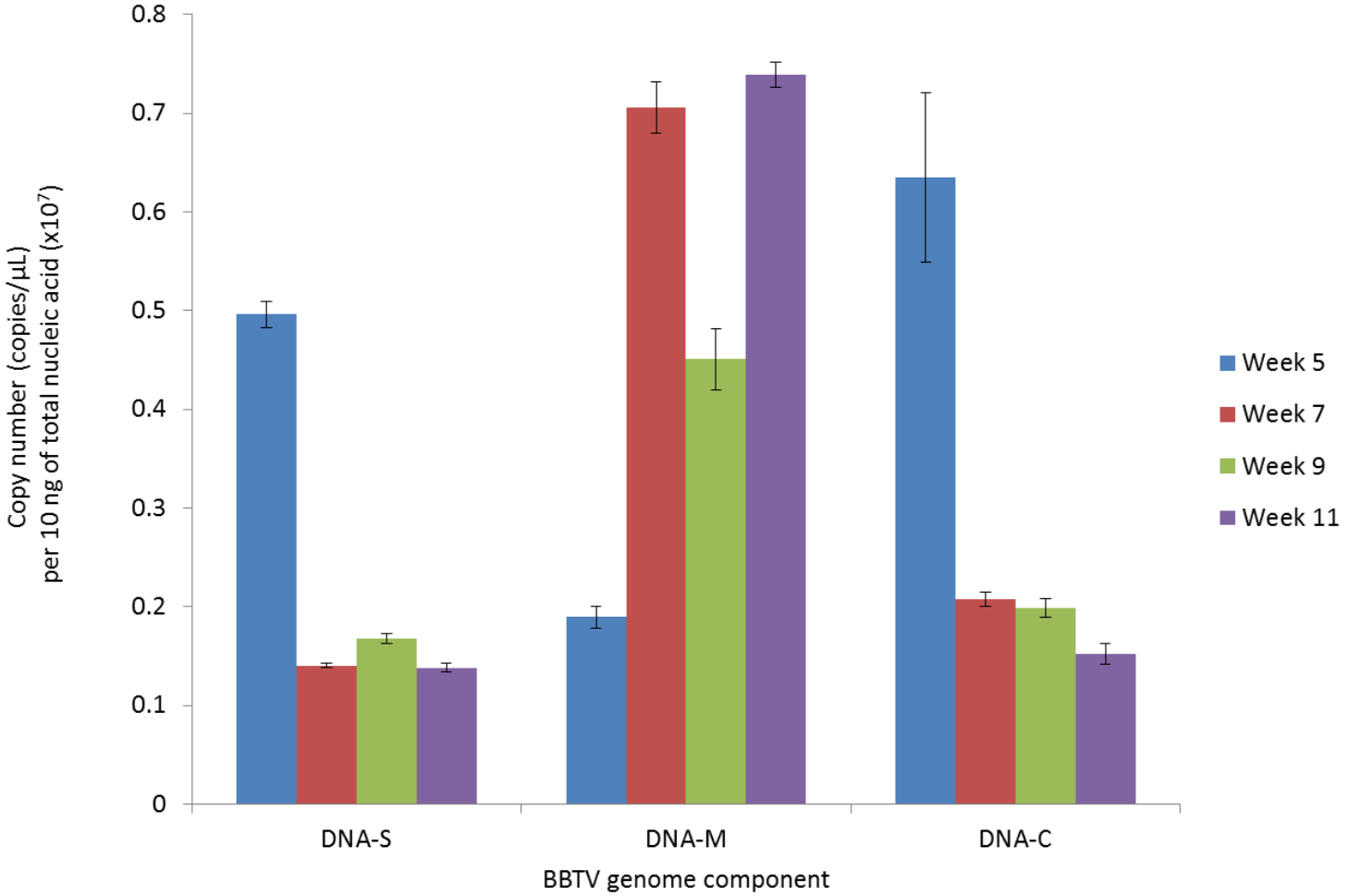


Figure 6.8: Magnified version of the qPCR results presented in Figure 6.7 excluding those for DNA-N, -R and -U3. Values shown are an average of three plants for each time point.

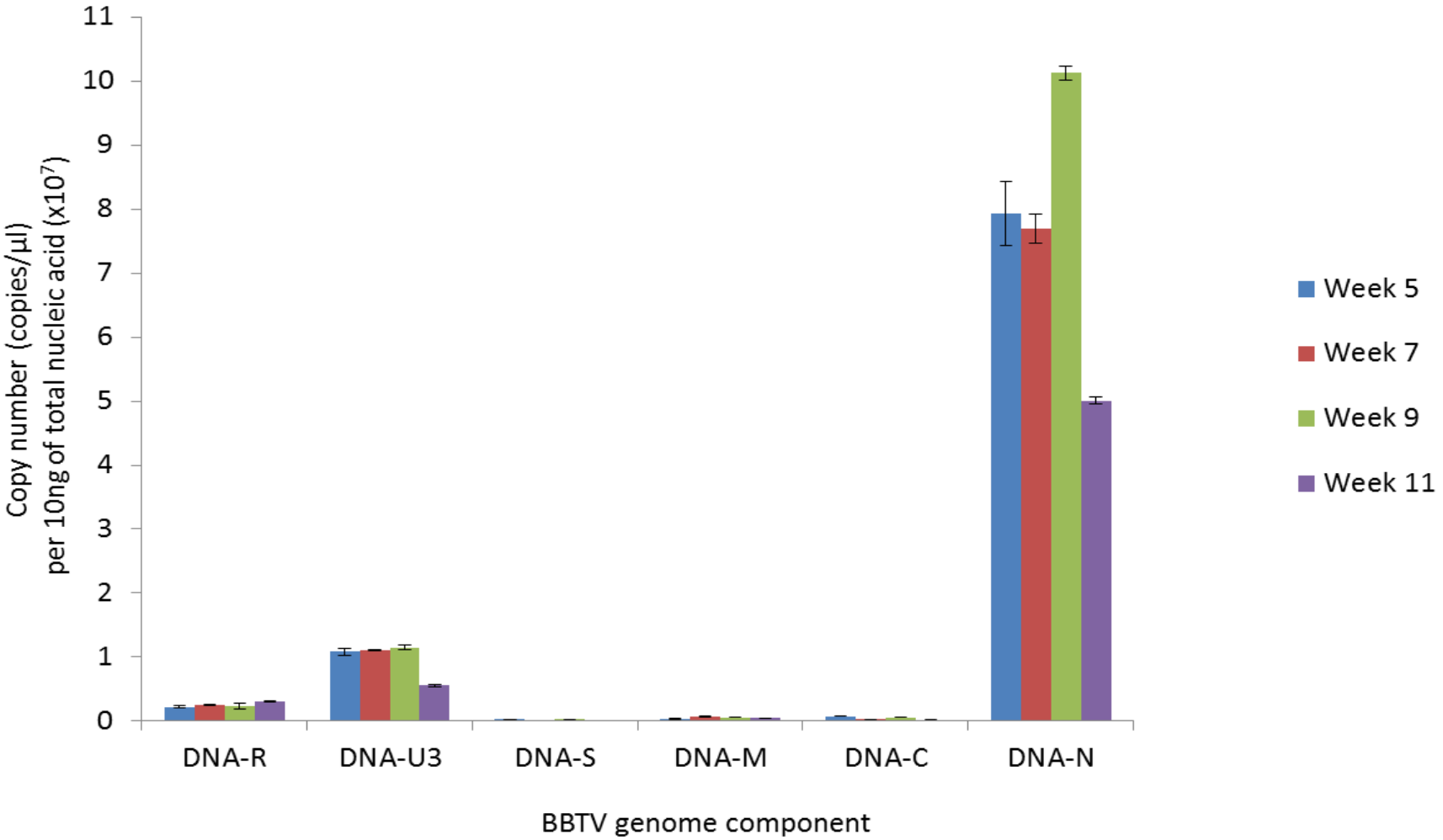


Figure 6.9: Absolute quantification of the six BBTV DNAs in BBTV-infected *Musa acuminata* ssp. *zebrina* plants at sampling intervals of two weeks from week 5 to 11 post-inoculation. Values shown are an average of three plants for each time point. BBTV DNA-N showed the highest absolute copy numbers across the different time points of sampling.

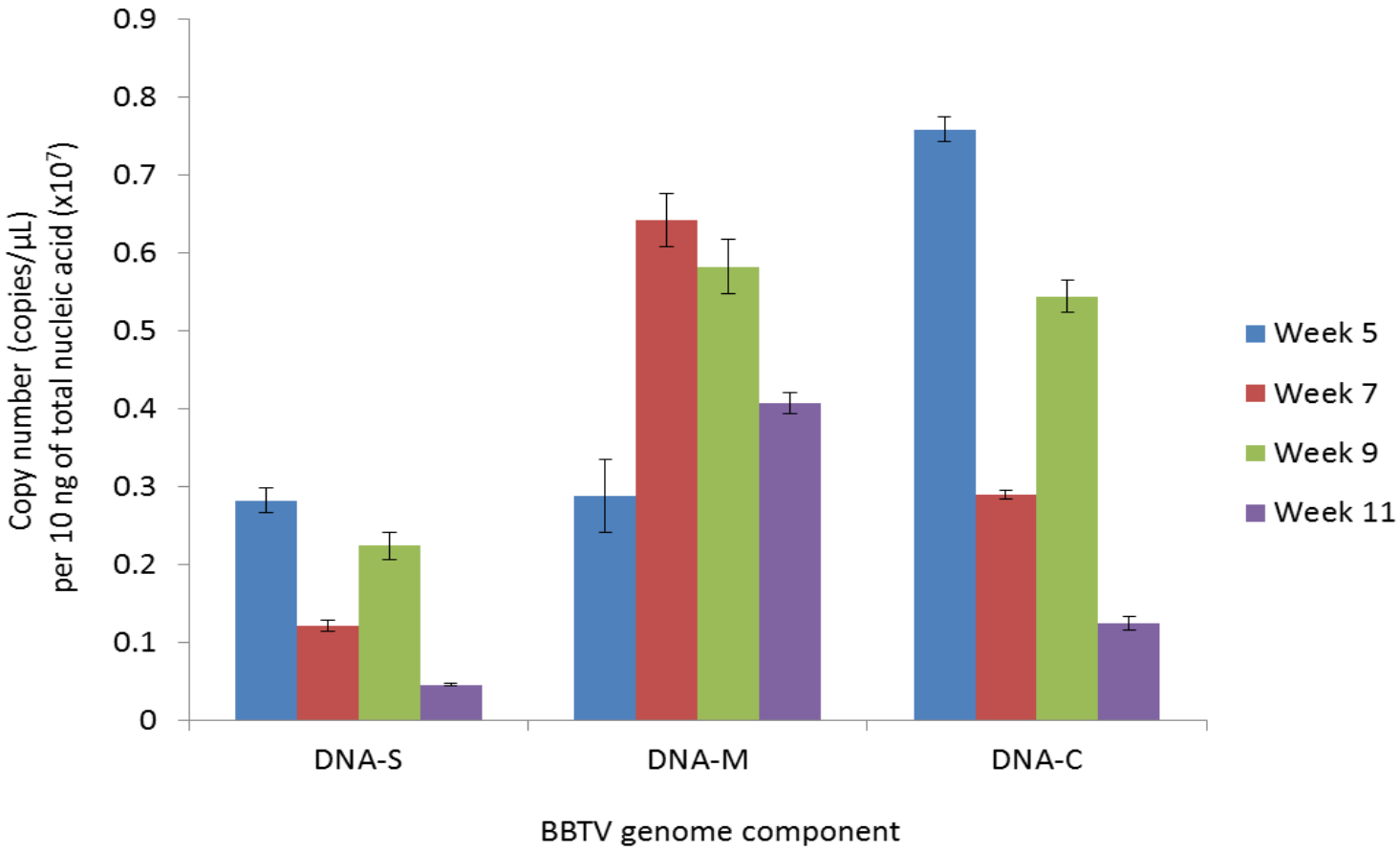


Figure 6.10: Magnified version of the qPCR results presented in Figure 6.9 excluding those for DNA-N, -R and -U3. Values shown are an average of three plants for each time point.

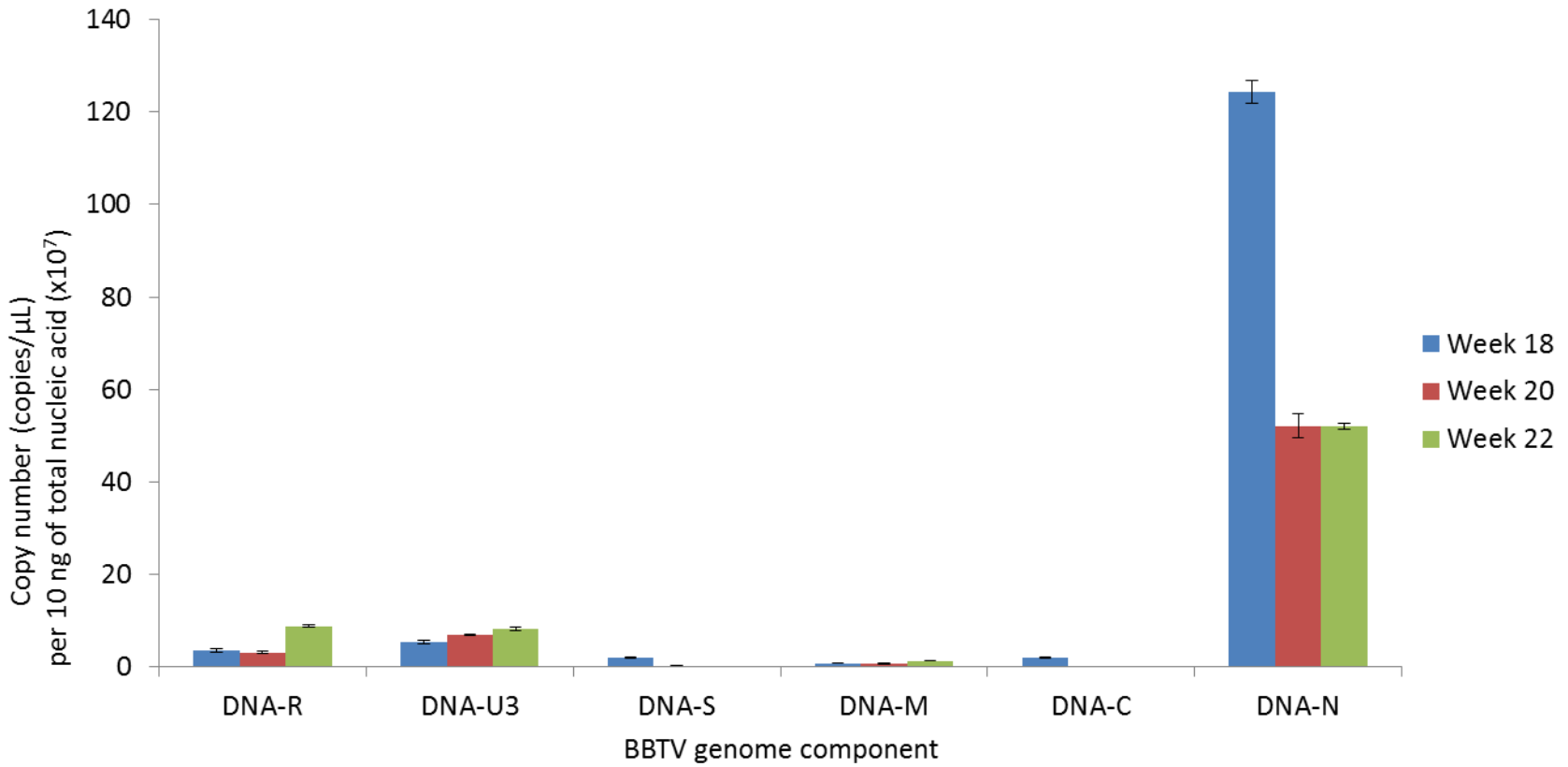


Figure 6.11: Absolute quantification of the six BBTV DNAs in BBTV-infected Pisang Gajih Merah at sampling intervals of two weeks from week 18 to 22 post-inoculation. Values shown are an average of three plants for each time point. BBTV DNA-N showed the highest absolute copy numbers across the different time points of sampling.

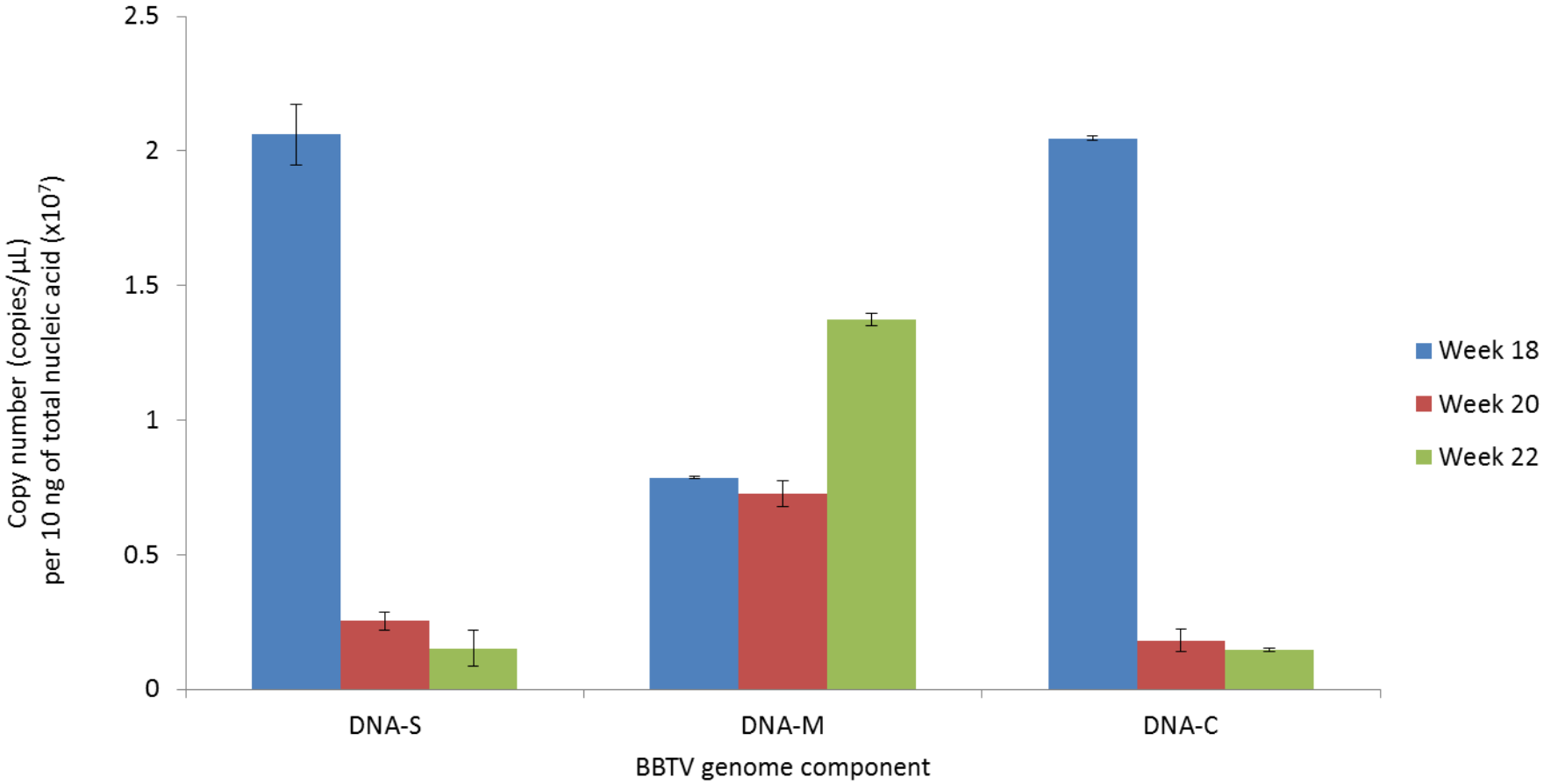


Figure 6.12: Magnified version of the qPCR results presented in Figure 6.11 excluding those for DNA-N, -R and -U3. Values shown are an average of three plants for each time point.

6.3.3 BBTV genome formula

To determine the genome formula of BBTV in infected banana plants, the relative level of each genomic component was calculated in the four cultivars tested based on the average relative accumulation of each viral DNA, with the component having the lowest accumulation ascribed a value of 1.

Relative accumulation analyses revealed that DNA-S was the least abundant genome component in all four cultivars, although at specific time points in specific cultivars, there were two occasions where DNA-M was the least abundant genome component (Table 6.3). In contrast, DNA-N was the most abundant component in all cultivars over the entire sampling period. When the average frequency over the four sampling time points was calculated for cultivar Dwarf Cavendish, for every copy of DNA-S there were two copies of DNA-C, 12 of DNA-M, 98 of DNA-U3, 108 of DNA-R and 1663 of DNA-N. Thus for Dwarf Cavendish a genome formula of ^{1}S ^{2}C ^{12}M ^{98}U3 ^{108}R ^{1663}N is proposed where numbers indicate the mean copy number of each virus DNA, rounded to the nearest integer, relative to that of the least abundant component (DNA-S). Similarly, for Lady Finger the genome formula (based on average values of four time points) was ^{1}S ^{1}C ^{3}M ^{18}R ^{34}U3 ^{287}N, for *Musa acuminata* ssp. *zebrina* the genome formula (based on average values of four time points) was ^{1}S ^{3}C ^{4}M ^{26}R ^{75}U3 ^{614}N and for Pisang Gajih Merah the genome formula (based on average values of three time points) was ^{1}S ^{1}C ^{4}M ^{24}R ^{28}U3 ^{203}N (Table 6.4). Interestingly, for Dwarf Cavendish, DNA-R was slightly higher (on average) compared to DNA-U3, whereas in the other three cultivars DNA-U3 was higher. Otherwise, the order of the components was consistent between the four cultivars assessed. Based on the genome formula calculated for each of the four cultivars, an average BBTV genome formula was then calculated, based on all sampling time points included, to be ^{1}S ^{2}C ^{6}M ^{44}R ^{59}U3 ^{692}N (Table 6.4).

Table 6.4: Relative accumulation and average levels of BBTV genome components in four different banana genotypes

Cultivar	**Relative frequency of each BBTV genome component**						
	Week	**DNA-R**	**DNA-U3**	**DNA-S**	**DNA-M**	**DNA-C**	**DNA-N**
Dwarf Cavendish	5	21	79	1	2	4	467
	7	36	93	1	6	1	695
	9	25	67	1	5	1	557
	11	351	151	1	36	1	4931
(Average)		**108**	**98**	**1**	**12**	**2**	**1663**
Lady Finger	5	1	8	1	0.4	1	75
	7	17	61	1	5	1	329
	9	16	26	1	3	1	345
	11	39	41	1	5	1	397
(Average)		**18**	**34**	**1**	**3**	**1**	**287**
Musa acuminata ssp. *zebrina*	5	8	38	1	1	3	281
	7	21	91	1	5	2	633
	9	10	51	1	3	2	452
	11	65	121	1	9	3	1091
(Average)		**26**	**75**	**1**	**4**	**3**	**614**
Pisang Gajih Merah	18	2	3	1	0.4	1	60
	20	12	28	1	3	1	206
	22	58	54	1	9	1	342
(Average)		**24**	**28**	**1**	**4**	**1**	**203**
Overall average		**44**	**59**	**1**	**6**	**2**	**692**

BBTV DNA-N was determined as the most abundant and several fold highest in accumulation independent of banana genotype and sampling time.

6.4 Discussion

This study investigated the response of a diverse collection of banana genotypes to BBTV infection under glasshouse conditions. Of the genotypes which developed symptoms, most were very characteristic of bunchy top including chlorotic leaf margins, narrowing and bunching of newly formed leaves and general stunting. The characteristic dark green streaks found along petioles were pronounced in Dwarf Cavendish, Pisang Gajih Merah, and Lady Finger. However, this symptom was rarely observed in Akondro Mainty except for faint streaks. *Musa acuminata* ssp. *zebrina*, Pisang Bankahulu, Akondro Mainty and Lady Finger generally displayed symptoms that manifested as chlorotic leaf margins that, over time, spread towards the midrib (Fig 6.2).

Although bunchy top symptoms are generally regarded as being highly diagnostic for BBTV, all plants were still tested for the virus by PCR. Apart from one symptomless Ney Poovan plant, there was a 100% correlation between the presence of symptoms and a positive PCR result. The results of PCR testing showed that bunchy top incidence was variable among different banana genotypes. As expected, all of the highly susceptible Dwarf Cavendish (AAA) control plants included in both experiments became infected. The infection rate for the Lady Finger (AAB) and *M. acuminata* ssp. *zebrina* (AA) cultivars was found to be 80%, while Pisang Bankahulu (AA) and Akondro Mainty had a 60% infection rate. Only 43% of Pisang Gajih Merah (ABB) plants became infected. Interestingly, unlike most other susceptible cultivars which usually displayed symptoms as early as three weeks post-inoculation, plants of this genotype displayed a delayed disease emergence with the first plant not showing symptoms until 18 weeks post-inoculation. Of the 19 Gros Michel (AAA) plants tested, only one plant developed symptoms and these were not evident until 12 weeks post-inoculation. In the study by Niyongere *et al.* (2011), no infection was detected in this cultivar. Saba (ABB), Butuhan (BB) and Khae Phrae (AA) remained virus-free during the entire screening period. Although B genome-containing cultivars such as Saba (ABB) and Butuhan (BB) have been shown to remain BBTV-free under field conditions (Niyongere *et al.*, 2011), this is the first report of a diploid AA (Khae Phrae) cultivar showing apparent

immunity to BBTV infection. While none of the 10 inoculated Ney Poovan (AB) plants showed symptoms, one tested positive for BBTV by PCR. Previous studies (Niyongere *et al.*, 2011; Niyongere *et al.*, 2013) have shown that B genome banana genotypes are generally more tolerant to BBTV infection. However, in the current study the presence of a B genome did not appear to confer greater protection to Pisang Gajih Merah and Lady Finger which had a 43% and 80% disease incidence, respectively. Further study could help to shed light on the differences observed here between the susceptible and tolerant B genome-containing cultivars.

It is believed that modern day interspecific triploids may have resulted from one or more steps of recombination and/or swapping of chromosomal segments between the A- and B-genomes (De Langhe *et al.*, 2010; Jeridi *et al.*, 2011; Perrier *et al.*, 2009). Thus, the majority, if not all, *Musa* cultivars may possess genomes consisting of different segments of the A- and B-genome although their classification does not reflect the diverse segments that may be integrated in the different cultivars. It is thought that this kind of hybridisation process among *M. acuminata* species may have contributed to the evolution of the edible AA and AAA types. One of the effects of these recombination events is that the resulting hybrid genomes may contain a disproportionate number of A- and B-genome alleles (Henry *et al.*, 2011). Thus, the response of Gros Michel and Khae Phrae to BBTV infection may be due to the presence of unknown segments derived from B-genome chromosomes during the hybridisation process.

Although further testing of the cultivars used in this study is needed, the results suggest they could be candidates for molecular characterisation to determine the factors influencing their response to BBTV infection. The finding that Khae Phrae (AA) remained disease free and only 1/19 Gros Michel (AAA) became infected is in contrast to the commonly held view that cultivars containing some B genome have greater tolerance to BBTV infection and raises a number of questions. Are there host- or cultivar-related morphological factors hindering aphids from efficiently feeding on some cultivars thereby reducing the chances of virus transmission? If this is the case in certain cultivars, could a study on aphid oviposition and feeding study

on the different cultivars help identify cultivars which are less preferred by the banana aphids? If indeed interspecific exchange of virus tolerance factors from B genomes to A genome cultivars may have occurred during interspecific hybridisation, a study to characterise these factors may help elucidate the basis of the response exhibited by the different cultivars to BBTV infection. A study to determine micro RNA and gene expression fluctuation and abundance pre- and post-virus infection may reveal candidate genes for banana improvement. Alternatively, a study to assess R gene homologs from the banana genotypes reported here as showing tolerance or immunity to BBTV infection may also necessitate the identification of candidate genes for virus disease resistance.

When rtPCR analyses were performed on DNA from different BBTV infected cultivars (Dwarf Cavendish (AAA), Lady Finger (AAB), *Musa acuminata* ssp. *zebrina* (AA) and Pisang Gajih Merah (ABB) to determine the absolute level of BBTV accumulation, DNA-N was revealed as the most abundant during BBTV infection cycle in all the cultivars at all sampling points. DNA-R and U3 were the second most abundant after DNA-N. DNA-S, -M and -C were the least abundant and were not significantly different from each other ($P<0.0001$) regardless of the sampling time and cultivar tested. In a recent study by Sicard *et al.* (2013) to determine the relative abundance and frequency of *Faba bean necrotic stunt virus* (FBNSV) DNA components in Faba bean plants, DNA-N and DNA-U4 were the most abundant during virus infection cycle with DNA-U4 found to accumulate to the highest level. In the present study, BBTV DNA-N accumulated to the highest level followed by DNA-U3. Like BBTV-U3, FBNSV DNA-U4 encodes a gene of unknown function (Grigoras *et al.*, 2009). It follows that the genes encoded by these two DNAs, as well as DNA-N, may have a critical role during the virus infection cycle.

Based on the results of qPCR analysis in this study, a genome formula of BBTV has been proposed for the first time. Although the absolute values for each component differed in the four genotypes assessed, the relative level of each component was more or less consistent with DNA-S, -C and –M being much lower than the other three components and DNA-N consistently much higher than all other components.

When the relative DNA levels were adjusted to a baseline of 1 copy (using DNA-S which had the lowest value throughout) an average genome formula for BBTV was determined to be S^{1} C^{2} M^{6} R^{44} $U3^{59}$ N^{692}. This result is in contrast to the results of Sicard *et al.* (2013) where the genome formula for FBNSV was established as S^{1} R^{2} C^{3} M^{3} $U1^{7}$ $U2^{10}$ N^{13} $U4^{16}$. It is interesting that in both the viruses it is the DNA components with an unknown function that have one of the highest 'relative copy number'. It seems these genes may be very crucial in the virus life cycle. It is also puzzling that there is a large order of magnitude difference in the relative amount of BBTV DNA-N compared to the other components (more than 10-fold higher), whereas in FBNSV the ratios are all in the order of 1-10 fold difference. Whether this is a host driven factor or inbuilt within each virus to moderate their gene ratios with such a striking difference is another opportunity for further exploration.

Diagnosis of plant viruses commonly targets conserved genome sequences based on consensus from alignment analyses or degenerate sequences (Desingu *et al.*, 2015; Legarreta *et al.*, 2000; Mason *et al.*, 2008). The reliability of a virus detection system is central to effective virus indexing and the generation of virus free germplasm (Saponari *et al.*, 2013). In BBTV, DNA-R has been the target in BBTV detection as it is one of the most conserved genomic components and plays a critical role in virus replication. Primers are often designed from the DNA-R sequences for BBTV PCR diagnosis (Hafner *et al.*, 1995). In the current study, DNA-N was shown to be the most abundant component during the life cycle of the virus infection in different banana cultivars. DNA-N is also one of the most conserved BBTV genomic components but has not been targeted for virus diagnosis. Based on the current findings, DNA-N may be a more appropriate target for BBTV detection.

CHAPTER 7

GENERAL DISCUSSION

Bananas are the most important fruit crop and are grown worldwide as a staple food source. Their production, however, is threatened by a variety of diseases caused by pathogens including viruses, fungi and bacteria. *Banana bunchy top virus* (BBTV) is the most devastating of the viruses to infect the plant causing distinctive bunching symptoms and often complete yield loss. Traditional breeding for the improvement of bananas is both time consuming and difficult due to the parthenocarpic nature of the plant. As such, genetic modification of preferred cultivars is likely the best means of improving common varieties for resistance to biotic and abiotic stresses and enhanced fruit characteristics. Recent studies using a local Indian variety of banana have shown that an RNAi approach, targeting the Master Rep gene (DNA-R) of BBTV, can confer resistance to the virus (Shekhawat *et al.*, 2012). In that study, two complementary Rep-derived sequences (capable of forming a hairpin) were spaced by a spliceable intron and constitutively expressed *in planta*. In conjunction with an early plant screening strategy to eliminate low-expressing transformants, 100% resistance against BBTV was reported in all plants challenged with the virus. This would suggest RNAi is an effective means of generating BBTV resistance in banana and that this strategy may be adaptable to control banana bunchy top disease in other preferred banana cultivars.

The main aim of this PhD study, therefore, was to genetically modify Australian dessert banana varieties (both Dwarf Cavendish and Grand Nain) with a suite of RNAi constructs targeting three different BBTV genes, namely the Master Rep (DNA-R), the movement protein (DNA-M) and the nuclear shuttle protein (DNA-N), to generate immunity to BBTV and determine the most appropriate target for robust transgenic resistance. A total of 10 hairpin constructs were assembled containing one, two or three gene hairpin sequences based on an Australian isolate of BBTV, and over 250 transgenic banana plants established *in vitro*. Due to the vast number of plants generated, only a subset of these transgenic lines could be challenged with BBTV using the natural aphid vector of the virus, *Pentalonia nigronervosa*, to

determine whether these plants were resistant to the virus. Of the three genes targeted, hairpins based on the BBTV DNA-M sequence provided the most effective resistance to BBTV infection with 7 of 11 lines tested showing complete immunity 12 weeks post infection with a maximum 25 % disease incidence in the remaining 4 lines. The DNA-M hairpin was also effective when co-expressed with a hairpin targeting the DNA-M gene sequence. In this case, 62 % of lines containing both hairpins showed complete immunity to the virus. Importantly, this action correlated strongly with (i) low copy number integration events, where single copy transgenic plants showed higher levels of resistance to high copy number events and (ii) the production of gene-specific, small 21 nt dsRNA species, hallmarks of the RNAi process. *Agrobacterium*-mediated transformation is the preferred method of gene transfer for the production of transgenic plants as it often results in low copy number events. Approximately 20 % of all plants generated in this study contained a single copy of the transgene and copy number was generally low, ranging between 1 and 6, which is consistent with other reports in banana (Borth *et al.*, 2011; Khanna *et al.*, 2004; Pérez-Hernández *et al.*, 2006). It is well known that transgene copy number and the site of integration can both strongly influence the expression level and the effectiveness of a transgene (Yang *et al.*, 2014; Zhang *et al.*, 2013). However, it is unclear why high copy number plants were less effective at conferring resistance to BBTV in this study. Perhaps complex multiple integrations may coincide with transgene re-arrangements or truncations resulting in aberrant hairpin RNA formation or alternatively these events may trigger methylation-associated transcriptional gene silencing resulting in the complete shutdown of hairpin expression. These findings contrast with those of Borth *et al.* (2011) who reported multiple transgene integration events in banana could confer effective resistance to BBTV infection.

Interestingly, the DNA-M gene product (MP) has recently been shown to be a major pathenogenicity determinant of BBTV and capable of suppressing post transcriptional gene silencing in *N. benthamiana* 16c transient assays (Amin et al., 2011). The ability of this protein to stabilise GFP-specific mRNA and reduce GFP-specific siRNAs suggests the BBTV MP may specifically bind small dsRNA species, in

a manner similar to the *Tomato bushy stunt virus* P19 silencing suppressor protein, thereby sequestering these RNAs from entering the RNA-Induced Silencing Complex (RISC) and preventing gene specific mRNA degradation (Amin et al., 2011). It is likely this strategy is key to BBTV to overcoming the host RNAi defence mechanism early in the infection process and is essential to prolonging the virus' lifecycle in banana. By targeting this early gene and constitutively over-expressing a hairpin directed toward the MP sequence in transgenic bananas, it is assumed that the abundant MP-specific siRNAs bind to and quench virus-encoded MP to block its suppressor activity. This in turn, may override the virus' defence mechanism ultimately providing host plant resistance. Considering the importance of the Rep gene in the BBTV lifecycle it is unclear why hairpins directed against this gene were not effective in providing resistance to BBTV in this study. In addition, this gene has been successfully targeted by other researchers for RNAi-based resistance to BBTV in transgenic bananas. Our results may simply reflect the small number of transgenic lines tested, their copy number or perhaps differences in hairpin design.

A useful RNAi-based control strategy for BBTV in transgenic bananas would provide resistance to geographically diverse strains of the virus which differ in homology at the nucleotide level. Based on sequence homology, there are two major strains of BBTV, those representing the South Pacific group (including Australia) and those representing the Asian group (including Vietnam). These groups are 85.5 % (DNA-M) and 90 % (DNA-R) similar to each other at the nucleotide level. Hairpins directed toward either the DNA-M or DNA-R genes from a Vietnamese isolate of BBTV failed to provide resistance to infection with the Australian strain of the virus. However, this finding was not conclusive considering the very small sample set tested (4 plants for DNA-R and 1 plant for DNA-M) and the high copy number of these lines (between 2-6 copies). It is likely that screening more transgenic lines will identify plants with some degree of broad spectrum resistance as the amount of homology between the two BBTV strains differs by less than 15 %. Other studies have shown an optimal response using RNAi is obtained with hairpins sharing at least 85 % nucleotide identity (Benedito *et al.*, 2004) and an effective RNAi response against the Pepper golden mosaic geminivirus was maintained with strains of the virus varying in nucleotide homology up to 15 % (Medina-Hernández *et al.*, 2013).

In this study, viruliferous aphids were successfully used to transmit BBTV and challenge transgenic lines and in most cases a 100 % infection rate was obtained with wildtype control plants. However, the rearing and maintenance of these insect vectors is a time consuming and challenging process. Fluctuations in growth conditions, particularly seasonal temperature changes, and the control of natural aphid predators can greatly affect population dynamics resulting in unreliable BBTV infection rates. BBTV cannot be mechanically transmitted and alternative artificial infection systems have remained largely unexplored for this virus. Recently, reports of successful artificial infection with nanovirus infectious DNA have been described (Grigoras *et al.*, 2009; Iranzo & Manrubia, 2012; Sicard *et al.*, 2013) suggesting this approach may be adaptable to BBTV in banana. Greater than component length forms of the six essential BBTV DNAs were generated and delivered into banana explants using either micro-projectile bombardment or *Agrobacterium*-mediated delivery. Despite optimising biolistic parameters, no infections could be established in banana with infectious BBTV DNAs or the control badnavirus, *Banana streak Mysore virus* (BSMV). In contrast, only BSMV infection could be established using *Agrobacterium*-mediated delivery. Together, this would suggest that the use of *Agrobacterium* as a vehicle for delivering infectious DNAs into banana may be the better of the two options, however, there are intrinsic differences between the two viruses that influence their ability to initiate an infection. BBTV may be limited by the fact that is a multicomponent virus whereby all six components are required in a single cell to establish an infection. Also, BBTV is a phloem-limited virus and, therefore, requires specialised phloem companion cells to replicate in and complete its life cycle. To overcome these limitations, infectious DNAs representing more than one component of the BBTV genome could be delivered on a single T-DNA to increase the frequency of co-transformation events and/or vacuum delivery of recombinant *Agrobacterium* cultures could be used to infiltrate small plantlets to maximise the amount of bacteria delivered into the plant and increase the probability of accessing the specialised target phloem cells.

Natural BBTV resistance has not been identified in the *Musa* germplasm, but it is believed that wild banana, *M. balbisiana* (B genotype), is a reservoir for genetic

resources, including abiotic and biotic resistance characteristics, that could be utilized in banana breeding programs (Wang *et al.*, 2007). In fact, recent field studies have shown a range of responses to BBTV infection in a diverse collection of banana genotypes (Niyongere *et al.*, 2011; Niyongere *et al.*, 2013). In the 2011 inoculation study, cultivars containing a B genome, with the exception of Gros Michel (AAA,) remained symptomless for 28 months following BBTV inoculation. In an effort to explore natural BBTV resistance in the *Musa* germplasm, 11 banana cultivars of diverse genotypes were inoculated with BBTV and monitored for disease symptoms. We identified B genome-containing varieties including Saba (ABB), Ney Poovan (AB) and A genome containing cultivar, Gros Michel (AAA) as tolerant to BBTV infection. However, Butuhan (BB) and Khae Phrae (AA) were completely immune to BBTV infection. This was the first time a diploid A wild type has been shown to be immune to BBTV infection and suggests the genes associated with BBTV resistance may not be confined to the *Musa* B genome. As advances in gene discovery evolve and new banana genomes are sequenced, these varieties may serve as a useful source of native banana genes for BBTV resistance. These genes and their upstream and downstream regulatory sequences could be particularly beneficial for the production of new cisgenic and disease resistant banana varieties.

It is well established that there are six essential DNA components that are readily detectable during a BBTV infection. However, until this point there was no evidence to suggest these components accumulate to different levels and follow a strict genomic formula governing their relative abundance throughout an infection, independent of banana genotype. Of the six BBTV components, DNA-N was by far the most over-represented whereas DNA-S and DNA-C were the least. It is unclear why this bias in component number occurs nor how it is attained and maintained over time. One may speculate that small nucleotide differences in key replication features of DNA-N, for example the iteron binding domains, may bind the Rep protein complex more efficiently thereby sequestering Rep from other DNA components. Alternatively, the architecture of the DNA-N intergenic region may favour accessibility of the replication complex. Perhaps the DNA-N gene product is essential for virus infection at all points of the infection and its expression must be

maintained at high levels throughout the process. Whether this genomic formula correlates with gene expression levels from each component could be easily determined using qRT-PCR to measure the expression levels of the six major genes at different time points during a BBTV infection. Whether this phenomenon is solely virus-directed or involves host factors, or both, remains to be determined.

In conclusion, this study has proven RNAi is an effective strategy for controlling BBTV in bananas, particularly the Cavendish dessert varieties of Australia. It will be of particular interest to determine whether this resistance is maintained in the field and over multiple generations. To this end, transgenic plants established during this PhD research will form part of a major field trial to be conducted over the coming years in Africa. Ultimately, these strategies will be adapted to other banana varieties to engineer BBTV resistance in farmer-preferred cultivars and eradicate the disease.

CHAPTER 8

REFERENCES

Abel, P. P., Nelson, R. S., De, B., Hoffmann, N., Rogers, S. G., Fraley, R. T., & Beachy, R. N. (1986). Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene. *Science, 232*(4751), 738-743.

Al Abdallat, M. A., Hmoud, S. D., Heba, A., Samar, M., Ayat, Q., & Anders, K. (2010). An efficient in vitro-inoculation method for Tomato yellow leaf curl virus. *Virology, 7*(1), 9.

Allen, R. N. (1987). Further studies on epidemiological factors influencing control of banana bunchy top disease and evaluation of control measures by computer simulation. *Australian Journal of Agricultural Research, 38*(2), 373-382.

Almeida, R. P. P., Bennett, G. M., Anhalt, M. D., Tsai, C. W., & O'Grady, P. (2009). Spread of an introduced vector-borne banana virus in Hawaii. *Molecular Ecology, 18*(1), 136-146.

Amin, I., Ilyas, M., Qazi, J., Bashir, R., Yadav, J. S., Mansoor, S., . and Briddon, R. W. (2011). Identification of a major pathogenicity determinant and suppressors of RNA silencing encoded by a South Pacific isolate of Banana bunchy top virus originating from Pakistan. *Virus Genes, 42*(2), 272-281.

Amin, I., Qazi, J., Mansoor, S., Ilyas, M., & Briddon, R. W. (2008). Molecular characterisation of Banana bunchy top virus (BBTV) from Pakistan. *Virus Genes, 36*(1), 191-198.

Anhalt, M. D., & Almeida, R. P. P. (2008). Effect of temperature, vector life stage, and plant access period on transmission of Banana bunchy top virus to banana. *Phytopathology, 98*(6), 743-748.

Bananej, K., Ghotbi, T., & Vahdat, A. (2007). First report of Banana bunchy top virus infecting banana in Iran. *Plant Pathology, 56*(4), 719.

Banerjee, A., Roy, S., Behere, G. T., Roy, S. S., Dutta, S. K., & Ngachan, S. V. (2014). Identification and characterization of a distinct banana bunchy top virus isolate of Pacific-Indian Oceans group from North-East India. *Virus Research, 183*, 41-49.

Baulcombe, D. C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. *Plant Cell, 8*(10), 1833-1844.

Baulcombe, D. C. (1999). Fast forward genetics based on virus-induced gene silencing. *Current Opinion in Plant Biology, 2*(2), 109-113.

Beetham, P. R., Hafner, G. J., Harding, R. M., & Dale, J. L. (1997). Two mRNAs are transcribed from banana bunchy top virus DNA-1. *Journal of General Virology, 78*(1), 229-236.

Bejarano, E. R., & Lichtenstein, C. P. (1994). Expression of TGMV antisense RNA in transgenic tobacco inhibits replication of BCTV but not ACMV geminiviruses. *Plant Molecular Biology, 24*(1), 241-248.

Bell, K. E., Dale, J. L., Ha, C. V., Vu, M. T., & Revill, P. A. (2002). Characterisation of Rep-encoding components associated with banana bunchy top nanovirus in Vietnam. *Archives of Virology, 147*(4), 695-707.

Bendahmane, M., & Gronenborn, B. (1997). Engineering resistance against tomato yellow leaf curl virus (TYLCV) using antisense RNA. *Plant Molecular Biology, 33*(2), 351-357.

Benedito, V. A., Visser, P. B., Angenent, G. C., & Krens, F. A. (2004). The potential of virus-induced gene silencing for speeding up functional characterization of plant genes. *Genetics and Molecular Research, 3*(3), 323-341.

Bjartan, P. K. B. (2012). Optimisation of Banana streak virus (BSV) diagnostic assays. Thesis submitted for the degree of Master of Science in the Centre for Tropical Crops and Biocommodities, Queensland University of Technology, Australia.

Bonilla-Ramírez, G. M., Guevara-González, R. G., Garzón-Tiznado, J. A., Ascencio-Ibáñez, J. T., Torres-Pacheco, I., & Rivera-Bustamante, R. F. (1997). Analysis of the infectivity of monomeric clones of pepper huasteco virus. *Journal of General Virology, 78*(4), 947-951.

Boonruangrod, R., Fluch, S., & Burg, K. (2009). Elucidation of origin of the present day hybrid banana cultivars using the 5'ETS rDNA sequence information. *Molecular Breeding, 24*(1), 77-91.

Borth, W., Perez, E., Cheah, K., Chen, Y., Xie, W. S., Gaskill, D., . and Hu, J. S. (2011) Transgenic banana plants resistant to Banana Bunchy top virus infection. The Journal of general virology. *Vol. 897* (pp. 449-458).

Briddon, R. W., Mansoor, S., Bedford, I. D., Pinner, M. S., Saunders, K., Stanley, J., . and Markham, P. G. (2001). Identification of DNA components required for induction of cotton leaf curl disease. *Virology, 285*(2), 234-243.

Bucher, E., Lohuis, D., van Poppel, P. M. J. A., Geerts-Dimitriadou, C., Goldbach, R., & Prins, M. (2006). Multiple virus resistance at a high frequency using a single transgene construct. *Journal of General Virology, 87*(12), 3697-3701.

Burns, T. M., Harding, R. M., & Dale, J. L. (1995). The genome organization of banana bunchy top virus: Analysis of six ssDNA components. *Journal of General Virology, 76*(6), 1471-1482.

Chellappan, P., Masona, M., Vanitharani, R., Taylor, N., & Fauquet, C. (2004). Broad Spectrum Resistance to ssDNA Viruses Associated with Transgene-Induced Gene Silencing in Cassava. *Plant Molecular Biology, 56*(4), 601-611.

Chellappan, P., Vanitharani, R., & Fauquet, C. M. (2004). Short interfering RNA accumulation correlates with host recovery in DNA virus-infected hosts, and gene silencing targets specific viral sequences. *Journal of Virology, 78*(14), 7465-7477.

Chen, Y. K., Lohuis, D., Goldbach, R., & Prins, M. (2004). High frequency induction of RNA-mediated resistance against Cucumber mosaic virus using inverted repeat constructs. *Molecular Breeding, 14*(3), 215-226.

Christelová, P., Valárik, M., Hřibová, E., Van den houwe, I., Channelière, S., Roux, N., & Doležel, J. (2011). A platform for efficient genotyping in musa using microsatellite markers. *AoB PLANTS, 11*(1).

Cooper, B., Lapidot, M., Heick, J. A., Dodds, J. A., & Beachy, R. N. (1995). A defective movement protein of TMV in transgenic plants confers resistance to multiple viruses whereas the functional analog increases susceptibility. *Virology, 206*(1), 307-313.

D'Hont, A., Denoeud, F., Aury, J. M., Baurens, F. C., Carreel, F., Garsmeur, O., . and Wincker, P. (2012). The banana (Musa acuminata) genome and the evolution of monocotyledonous plants. *Nature, 488*(7410), 213-217.

Dale, J. L. (1987) Banana Bunchy Top: An Economically Important Tropical Plant Virus Disease. *Vol. 33* (pp. 301-325).

Davey, M. W., Gudimella, R., Harikrishna, J. A., Sin, L. W., Khalid, N., & Keulemans, J. (2013). " A draft Musa balbisiana genome sequence for molecular genetics in polyploid, inter- and intra-specific Musa hybrids". *BMC Genomics, 14*(1).

Davey, M. W., Van den Bergh, I., Markham, R., Swennen, R., & Keulemans, J. (2009). Genetic variability in Musa fruit provitamin A carotenoids, lutein and mineral micronutrient contents. *Food Chemistry, 115*(3), 806-813.

Day, A. G., Bejarano, E. R., Buck, K. W., Burrell, M., & Lichtenstein, C. P. (1991). Expression of an antisense viral gene in transgenic tobacco confers resistance to the DNA virus tomato golden mosaic virus. *Proceedings of the National Academy of Sciences of the United States of America, 88*(15), 6721-6725.

De Langhe, E., Hřibová, E., Carpentier, S., Doleel, J., & Swennen, R. (2010). Did backcrossing contribute to the origin of hybrid edible bananas? *Annals of Botany, 106*(6), 849-857.

De Langhe, E., Vrydaghs, L., De Maret, P., Perrier, X., & Denham, T. (2009). Why bananas matter: An introduction to the history of banana domestication. *Ethnobotany Research and Applications, 7*, 165-178.

Desingu, P. A., Singh, S. D., Dhama, K., Kumar, O. R. V., Singh, R., & Singh, R. K. (2015). A rapid method of accurate detection and differentiation of Newcastle disease virus pathotypes by demonstrating multiple bands in degenerate primer based nested RT-PCR. *Journal of Virological Methods, 212*, 47-52.

Di Nicola, E., Tavazza, M., Lucioli, A., Salandri, L., & Ilardi, V. (2014). Robust RNA silencing-mediated resistance to Plum pox virus under variable abiotic and biotic conditions. *Molecular Plant Pathology, 15*(8), 841-847.

Drucker, M., & Then, C. (2015). Transmission activation in non-circulative virus transmission: a general concept? *Current Opinion in Virology, 15*, 63-68.

Duan, C.-G., Wang, C.-H., Fang, R.-X., & Guo, H.-S. (2008). Artificial MicroRNAs Highly Accessible to Targets Confer Efficient Virus Resistance in Plants. *Journal of Virology, 82*(22), 11084-11095.

Duan, Y. P., Powell, C. A., Purcifull, D. E., Broglio, P., & Hiebert, E. (1997). Phenotypic variation in transgenic tobacco expressing mutated geminivirus movement/pathogenicity (BC1) proteins. *Molecular Plant-Microbe Interactions, 10*(9), 1065-1074.

Duan, Y. P., Powell, C. A., Webb, S. E., Purcifull, D. E., & Hiebert, E. (1997). Geminivirus resistance in transgenic tobacco expressing mutated BC1 protein. *Molecular Plant-Microbe Interactions, 10*(5), 617-623.

Ecker, J. R., & Davis, R. W. (1986). Inhibition of gene expression in plant cells by expression of antisense RNA. *Proceedings of the National Academy of Sciences of the United States of America, 83*(15), 5372-5376.

Eini, O., Dogra, S. C., Dry, I. B., & Randles, J. W. (2012). Silencing suppressor activity of a begomovirus DNA β encoded protein and its effect on heterologous helper virus replication. *Virus Research, 167*(1), 97-101.

Elmer, J. S., Sunter, G., Gardiner, W. E., Brand, L., Browning, C. K., Bisaro, D. M., & Rogers, S. G. (1988). Agrobacterium-mediated inoculation of plants with tomato golden mosaic virus DNAs. *Plant Molecular Biology, 10*(3), 225-234.

Englberger, L., Schierle, J., Aalbersberg, W., Hofmann, P., Humphries, J., Huang, A., . and Fitzgerald, M. H. (2006). Carotenoid and vitamin content of Karat and other Micronesian banana cultivars. *International Journal of Food Sciences and Nutrition, 57*(5-6), 399-418.

FAO. (2010). FAOSTAT provides time-series and cross sectional data relating to food and agriculture for some 200 countries. *For a world without hunger*, from http://faostat.fao.org/site/339/default.aspx

Fauquet, C. (2005) *Virus taxonomy: classification and nomenclature of viruses : eighth report of the International Committee on the Taxonomy of Viruses* (2 ed.): Elsevier Academic Press, 2005.

Fauquet, C. M., Bisaro, D. M., Briddon, R. W., Brown, J. K., Harrison, B. D., Rybicki, E. P., . and Stanley, J. (2003). Revision of taxonomic criteria for species demarcation in the family Geminiviridae, and an updated list of begomovirus species. *Archives of Virology, 148*(2), 405-421.

Ferreira, P. d. T. d. O., Lemos, T. O., Nagata, T., & Inoue-Nagata, A. K. (2008). One-step cloning approach for construction of agroinfectious begomovirus clones. *Journal of Virological Methods, 147*(2), 351-354.

Gaba, V., Rosner, A., Maslenin, L., Leibman, D., Singer, S., Kukurt, E., . and Gal-On, A. (2010). Hairpin-based virus resistance depends on the sequence similarity between challenge virus and discrete, highly accumulating siRNA species. *European Journal of Plant Pathology, 128*(2), 153-164.

Gaeta, R. T., & Pires, C. J. (2010). Homoeologous recombination in allopolyploids: The polyploid ratchet. *New Phytologist, 186*(1), 18-28.

Gleba, Y., Klimyuk, V., & Marillonnet, S. (2007). Viral vectors for the expression of proteins in plants. *Current Opinion in Biotechnology, 18*(2), 134-141.

Gleba, Y., Marillonnet, S., & Klimyuk, V. (2004). Engineering viral expression vectors for plants: the 'full virus' and the 'deconstructed virus' strategies. *Current Opinion in Plant Biology, 7*(2), 182-188.

Grigoras, I., Timchenko, T., Katul, L., Grande-Perez, A., Vetten, H. J., & Gronenborn, B. (2009). Reconstitution of authentic nanovirus from multiple cloned DNAs. *Journal of Virology, 83*(20), 10778-10787.

Grimsley, N., Hohn, T., Davies, J. W., & Hohn, B. (1987). Agrobacterium-mediated delivery of infectious maize streak virus into maize plants. *Nature, 325*(6100), 177-179.

Grumet, R., Sanford, J. C., & Johnston, S. A. (1987). Pathogen-derived resistance to viral infection using a negative regulatory molecule. *Virology, 161*(2), 561-569.

Guo, S., & Kemphues, K. J. (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. *Cell, 81*(4), 611-620.

Gutierrez, C. (1999). Geminivirus DNA replication. *Cellular and Molecular Life Sciences, 56*(3-4), 313-329.

Gutierrez, C. (2000). DNA replication and cell cycle in plants: Learning from geminiviruses. *EMBO Journal, 19*(5), 792-799.

Gutzat, R., Borghi, L., & Gruissem, W. (2012). Emerging roles of RETINOBLASTOMA-RELATED proteins in evolution and plant development. *Trends in Plant Science, 17*(3), 139-148.

Hafner, G. J., Harding, R. M., & Dale, J. L. (1995). Movement and transmission of banana bunchy top virus DNA component one in bananas. *Journal of General Virology, 76*(9), 2279-2285.

Hanley-Bowdoin, L., Settlage, S. B., Orozco, B. M., Nagar, S., & Robertson, D. (1999). Geminiviruses: Models for plant DNA replication, transcription, and cell cycle regulation. *Critical Reviews in Plant Sciences, 18*(1), 71-106.

Hanley-Bowdoin, L., Settlage, S. B., & Robertson, D. (2004). Reprogramming plant gene expression: A prerequisite to geminivirus DNA replication. *Molecular Plant Pathology, 5*(2), 149-156.

Hannon, G. J. (2002). RNA interference. *Nature, 418*(6894), 244-251.

Harding, R. M., Burns, T. M., & Dale, J. L. (1991). Virus-like particles associated with banana bunchy top disease contain small single-stranded DNA. *Journal of General Virology, 72*(2), 225-230.

Harish, S., Kavino, M., Kumar, N., Saravanakumar, D., Soorianathasundaram, K., & Samiyappan, R. (2008). Biohardening with Plant Growth Promoting Rhizosphere and Endophytic bacteria induces systemic resistance against Banana bunchy top virus. *Applied Soil Ecology, 39*(2), 187-200.

Henry, I. M., Carpentier, S. C., Pampurova, S., van Hoylandt, A., Panis, B., Swennen, R., & Remy, S. (2011). Structure and regulation of the Asr gene family in banana. *Planta, 234*(4), 785-798.

Herwig, S., & Strauss, M. (1997). The retinoblastoma protein: A master regulator of cell cycle, differentiation and apoptosis. *European Journal of Biochemistry, 246*(3), 581-601.

Heslop-Harrison, J. S., & Schwarzacher, T. (2007). Domestication, Genomics and the Future for Banana. *Annals of Botany, 100*(5), 1073-1084.

Hong, Y., & Stanley, J. (1996). Virus resistance in Nicotiana benthamiana conferred by African cassava mosaic virus replication-associated protein (AC1) transgene. *Molecular Plant-Microbe Interactions, 9*(4), 219-225.

Horser, C. L., Harding, R. M., & Dale, J. L. (2001). Banana bunchy top nanovirus DNA-1 encodes the 'master' replication initiation protein. *Journal of General Virology, 82*(2), 459-464.

Horser, C. L., Karan, M., Harding, R. M., & Dale, J. L. (2001). Additional Rep-encoding DNAs associated with banana bunchy top virus. *Archives of Virology, 146*(1), 71-86.

Hou, Y. M., Sanders, R., Ursin, V. M., & Gilbertson, R. L. (2000). Transgenic plants expressing geminivirus movement proteins: Abnormal phenotypes and delayed infection by Tomato mottle virus in transgenic tomatoes expressing the Bean dwarf mosaic virus BV1 or BC1 proteins. *Molecular Plant-Microbe Interactions, 13*(3), 297-308.

Hu, J. M., Fu, H. C., Lin, C. H., Su, H. J., & Yeh, H. H. (2007). Reassortment and concerted evolution in Banana bunchy top virus genomes. *Journal of Virology, 81*(4), 1746-1761.

Incarbone, M., & Dunoyer, P. (2013). RNA silencing and its suppression: novel insights from in planta analyses. *Trends in Plant Science, 18*(7), 382-392.

Iranzo, J., & Manrubia, S. C. (2012). Evolutionary dynamics of genome segmentation in multipartite viruses. *Proceedings of the Royal Society B: Biological Sciences, 279*(1743), 3812-3819.

Jagtap, U. B., Gurav, R. G., & Bapat, V. A. (2011). Role of RNA interference in plant improvement. *Naturwissenschaften, 98*(6), 473-492.

Jeridi, M., Bakry, F., Escoute, J., Fondi, E., Carreel, F., Ferchichi, A., . and Rodier-Goud, M. (2011). Homoeologous chromosome pairing between the A and B genomes of Musa spp. revealed by genomic in situ hybridization. *Annals of Botany, 108*(5), 975-981.

Jeske, H., Lütgemeier, M., & Preiß, W. (2001). DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus. *EMBO Journal, 20*(21), 6158-6167.

Jeske, H., Lutgemeier, M., & Preisz, W. (2001). DNA forms indicate rolling circle and recombination-dependent replication of Abutilon mosaic virus. *EMBO Journal, 20*(21), 6158-6167.

Kalantidis, K., Psaradakis, S., Tabler, M., & Tsagris, M. (2002). The occurrence of CMV-specific short RNAs in transgenic tobacco expressing virus-derived double-stranded RNA is indicative of resistance to the virus. *Molecular Plant-Microbe Interactions, 15*(8), 826-833.

Karan, M., Harding, R. M., & Dale, J. L. (1994). Evidence for two groups of banana bunchy top virus isolates. *Journal of General Virology, 75*(12), 3541-3546.

Karan, M., Robert M Harding, & Dale*, J. L. (1997). Association of Banana Bunchy Top Virus DNA Components 2 to 6 with Bunchy Top Disease. *Molecular Plant Pathology*, www.bspp.org.uk/mppol/.

Kasschau, K. D., & Carrington, J. C. (1998). A counterdefensive strategy of plant viruses: Suppression of posttranscriptional gene silencing. *Cell, 95*(4), 461-470.

Katul, L., Timchenko, T., Gronenborn, B., & Vetten, H. J. (1998). Ten distinct circular ssDNA components, four of which encode putative replication-associated proteins, are associated with the faba bean necrotic yellows virus genome. *Journal of General Virology, 79*(12), 3101-3109.

Kavino, M., Harish, S., Kumar, N., Saravanakumar, D., & Samiyappan, R. (2011). Molecular characterisation of coat protein and nuclear shuttle protein genes of banana bunchy top virus from western ghats in India. *Archives of Phytopathology and Plant Protection, 44*(5), 405-411.

Khanna, H., Becker, D., Kleidon, J., & Dale, J. (2004). Centrifugation Assisted Agrobacterium tumefaciens-mediated Transformation (CAAT) of embryogenic cell suspensions of banana (Musa spp. Cavendish AAA and Lady finger AAB). *Molecular Breeding, 14*(3), 239-252.

Kumar, P. L., Hanna, R., Alabi, O. J., Soko, M. M., Oben, T. T., Vangu, G. H. P., & Naidu, R. A. (2011). Banana bunchy top virus in sub-Saharan Africa: Investigations on virus distribution and diversity. *Virus Research, In Press, Corrected Proof*.

Kumar, P. L., Hanna, R., Alabi, O. J., Soko, M. M., Oben, T. T., Vangu, G. H. P., & Naidu, R. A. (2011). Banana bunchy top virus in sub-Saharan Africa: Investigations on virus distribution and diversity. *Virus Research, 159*(2), 171-182.

Kumar, P. L., Selvarajan, R., Iskra-Caruana, M. L., Chabannes, M., & Hanna, R. (2015) Biology, etiology, and control of virus diseases of banana and plantain. *Vol. 91. Advances in Virus Research* (pp. 226-269).

Kunik, T., Salomon, R., Zamir, D., Navot, N., Zeidan, M., Michelson, I., . and Czosnek, H. (1994). Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein are resistant to the virus. *Bio/Technology, 12*(5), 500-504.

Lapidot, M., Weil, G., Cohen, L., Segev, L., & Gaba, V. (2007). Biolistic inoculation of plants with Tomato yellow leaf curl virus DNA. *Journal of Virological Methods, 144*(1-2), 143-148.

Legarreta, G. G., García, M. L., Costa, N., & Grau, O. (2000). A highly sensitive heminested RT-PCR assay for the detection of citrus psorosis virus targeted to a conserved region of the genome. *Journal of Virological Methods, 84*(1), 15-22.

Liang, J., Deng, G., Long, H., Pan, Z., Wang, C., Cai, P., . and Yu, M. (2012). Virus-induced silencing of genes encoding LEA protein in Tibetan hulless barley (Hordeum vulgare ssp. vulgare) and their relationship to drought tolerance. *Molecular Breeding, 30*(1), 441-451.

Lindbo, J. A., & Dougherty, W. G. (2005). Plant Pathology and RNAi: A Brief History. *Annual Review of Phytopathology, 43*(1), 191-204.

Llave, C., Kasschau, K. D., & Carrington, J. C. (2000). Virus-encoded suppressor of posttranscriptional gene silencing targets a maintenance step in the silencing pathway. *Proceedings of the National Academy of Sciences of the United States of America, 97*(24), 13401-13406.

Lu, Y., Xu, W. H., Xie, Y. X., Zhang, X., Pu, J. J., Qi, Y. X., & Li, H. P. (2011). Isolation and characterization of nucleotide-binding site and C-terminal leucine-rich repeat-resistance gene candidates in bananas. *Genetics and molecular research : GMR, 10*(4), 3098-3108.

Magee, C. J. P. (1927). Investigation on the Bunchy Top Disease of the Banana. [CSIRO Bulletin]. 1-87.

Mahdavi, F., Mahmood, M., & Noor, N. M. (2014). Optimization of particle bombardment parameters for DNA delivery into the male flowers of banana. *Biologia (Poland), 69*(7), 888-894.

Mahendhiran, M., Ramirez-Prado, J. H., Escobedo-Gracia Medrano, R. M., Canto-Canché, B., Tzec-Simá, M., Grijalva-Arango, R., & James-Kay, A. (2014). Single nucleotide polymorphisms in partial sequences of the gene encoding the large sub-units of ADP-glucose pyrophosphorylase within a representative collection of 10 Musa genotypes. *Electronic Journal of Biotechnology, 17*(3), 137-147.

Mahmood ur, R., Ali, I., Husnain, T., & Riazuddin, S. (2008). RNA interference: The story of gene silencing in plants and humans. *Biotechnology Advances, 26*(3), 202-209.

Malik, A. H., Briddon, R. W., & Mansoor, S. (2011). Infectious clones of Tomato leaf curl Palampur virus with a defective DNA B and their pseudo-recombination with Tomato leaf curl New Delhi virus. *Virology journal, 8*(173), 10.1186/1743-1422X-1188-1173.

Mandal, B. (2010). Advances in small isometric multicomponent ssDNA viruses infecting plants. *Indian Journal of Virology, 21*(1), 18-30.

Maruthi, M. N., Bouvaine, S., Tufan, H. A., Mohammed, I. U., & Hillocks, R. J. (2014). Transcriptional Response of Virus-Infected Cassava and Identification of Putative Sources of Resistance for Cassava Brown Streak Disease. *PLoS ONE, 9*(5), 1-9.

Mason, G., Caciagli, P., Accotto, G. P., & Noris, E. (2008). Real-time PCR for the quantitation of Tomato yellow leaf curl Sardinia virus in tomato plants and in Bemisia tabaci. *Journal of Virological Methods, 147*(2), 282-289.

Medina-Hernández, D., Rivera-Bustamante, R. F., Tenllado, F., & Holguín-Peña, R. J. (2013). Effects and effectiveness of two RNAi constructs for resistance to pepper golden mosaic virus in Nicotiana benthamiana plants. *Viruses, 5*(12), 2931-2945.

Meister, G., & Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. *Nature, 431*(7006), 343-349.

Momonoi, K., Mori, M., Matsuura, K., Moriwaki, J., & Morikawa, T. (2015). Quantification of Mirafiori lettuce big-vein virus and its vector, Olpidium virulentus, from soil using real-time PCR. *Plant Pathology, 64*(4), 825-830.

Montes, C., Castro, Á., Barba, P., Rubio, J., Sánchez, E., Carvajal, D., . and Prieto, H. (2014). Differential RNAi responses of Nicotiana benthamiana individuals transformed with a hairpin-inducing construct during Plum pox virus challenge. *Virus Genes, 49*(2), 325-338.

Nakamura, H., Shin, M.-R., Fukagawa, T., Arita, M., Mikami, T., & Kodama, H. (2014). A tobacco calmodulin-related protein suppresses sense transgene-induced RNA silencing but not inverted repeat-induced RNA silencing. *Plant Cell, Tissue and Organ Culture (PCTOC), 116*(1), 47-53.

Niu, S., Wang, B., Guo, X., Yu, J., Wang, X., Xu, K., . and Liu, Z. (2009). Identification of two RNA silencing suppressors from banana bunchy top virus. *Archives of Virology, 154*(11), 1775-1783.

Niyongere, C., Ateka, E., Losenge, T., Blomme, G., & Lepoint, P. (2011) Screening Musa genotypes for banana bunchy top disease resistance in Burundi. *Vol. 897. Acta Horticulturae* (pp. 439-448).

Niyongere, C., Losenge, T., Ateka, E. M., Ntukamazina, N., Ndayiragije, P., Simbare, A., . and Blomme, G. (2013). Understanding banana bunchy top disease epidemiology in Burundi for an enhanced and integrated management approach. *Plant Pathology, 62*(3), 562-570.

Noris, E., Accotto, G. P., Tavazza, R., Brunetti, A., Crespi, S., & Tavazza, M. (1996). Resistance to tomato yellow leaf curl geminivirus in Nicotiana benthamiana plants transformed with a truncated viral C1 gene. *Virology, 224*(1), 130-138.

Novák, P., Hřibová, E., Neumann, P., Koblížková, A., Doležel, J., & Macas, J. (2014). Genome-wide analysis of repeat diversity across the family musaceae. *PLoS ONE, 9*(6), e98918.

Ortiz, R. (2013) Conventional banana and plantain breeding. *Vol. 986. Acta Horticulturae* (pp. 177-194).

Pakniat-Jahromy, A., Behjatnia, S. A. A., Dry, I. B., Izadpanah, K., & Rezaian, M. A. (2010). A new strategy for generating geminivirus resistant plants using a

DNA betasatellite/split barnase construct. *Journal of Virological Methods, 170*(1-2), 57-66.

Pérez-Hernández, J., Swennen, R., & Sági, L. (2006). Number and Accuracy of T-DNA Insertions in Transgenic Banana (<i>Musa</i> spp.) Plants Characterized by an Improved Anchored PCR Technique. *Transgenic Research, 15*(2), 139-150.

Perrier, X., Bakry, F., Carreel, F., Jenny, C., Horry, J. P., Lebot, V., & Hippolyte, I. (2009). Combining biological approaches to shed light on the evolution of edible bananas. *Ethnobotany Research and Applications, 7*, 199-216.

Pilartz, M., & Jeske, H. (2003). Mapping of Abutilon mosaic geminivirus minichromosomes. *Journal of Virology, 77*(20), 10808-10818.

Pradhan, B., Naqvi, A. R., Saraf, S., Mukherjee, S. K., & Dey, N. (2015). Prediction and characterization of Tomato leaf curl New Delhi virus (ToLCNDV) responsive novel microRNAs in Solanum lycopersicum. *Virus Research, 195*, 183-195.

Pumplin, N., & Voinnet, O. (2013). RNA silencing suppression by plant pathogens: Defence, counter-defence and counter-counter-defence. *Nature Reviews Microbiology, 11*(11), 745-760.

Raboin, L. M., Carreel, F., Noyer, J. L., Baurens, F. C., Horry, J. P., Bakry, F., . and Lagoda, P. J. L. (2005). Diploid ancestors of triploid export banana cultivars: Molecular identification of 2n restitution gamete Donors and n gamete donors. *Molecular Breeding, 16*(4), 333-341.

Ramesh, S. V., Mishra, A. K., & Praveen, S. (2007). Hairpin RNA-Mediated Strategies for Silencing of Tomato Leaf Curl Virus AC1 and AC4 Genes for Effective Resistance in Plants. *Oligonucleotides, 17*(2), 251-257.

Register, J. C., & Beachy, R. N. (1988). Resistance to TMV in transgenic plants results from interference with an early event in infection. *Virology, 166*(2), 524-532.

Robson, J. D., Wright, M. G., & Almeida, R. P. P. (Writers). (2006). Within-Plant Distribution and Binomial Sampling of Pentalonia nigronervosa (Hemiptera: Aphididae) on Banana, *Journal of Economic Entomolology 99(6), 2185-2190.*

Rodríguez-Negrete, E., Lozano-Durán, R., Piedra-Aguilera, A., Cruzado, L., Bejarano, E. R., & Castillo, A. G. (2013). Geminivirus Rep protein interferes with the plant DNA methylation machinery and suppresses transcriptional gene silencing. *New Phytologist, 199*(2), 464-475.

Roth, B. M., Pruss, G. J., & Vance, V. B. (2004). Plant viral suppressors of RNA silencing. *Virus Research, 102*(1), 97-108.

Rothstein, S. J., DiMaio, J., Strand, M., & Rice, D. (1987). Stable and heritable inhibition of the expression of nopaline synthase in tobacco expressing antisense RNA. *Proceedings of the National Academy of Sciences of the United States of America, 84*(23), 8439-8443.

Rouhibakhsh, A., Haq, Q. M. I., & Malathi, V. G. (2011). Mutagenesis in ORF AV2 affects viral replication in Mungbean yellow mosaic India virus. *Journal of Biosciences, 36*(2), 329-340.

Sambrook, J., & Russell, M. G. (2012). Molecular cloning : a laboratory manual. *Cold Spring Harbor Laboratory Press, c2012. , Fourth Edition*, 2029.

Sanford, J. C., & Johnston, S. A. (1985). The concept of parasite-derived resistance—Deriving resistance genes from the parasite's own genome. *Journal of Theoretical Biology, 113*(2), 395-405.

Sangaré, A., Deng, D., Fauquet, C. M., & Beachy, R. N. (1999). Resistance to African cassava mosaic virus conferred by a mutant of the putative NTP-binding domain of the Rep gene (AC1) in Nicotiana benthamiana. *Molecular Breeding, 5*(2), 95-102.

Saponari, M., Loconsole, G., Liao, H.-H., Jiang, B., Savino, V., & Yokomi, R. K. (2013). Validation of high-throughput real time polymerase chain reaction assays for simultaneous detection of invasive citrus pathogens. *Journal of Virological Methods, 193*(2), 478-486.

Scholthof, H. B. (2006). The Tombusvirus-encoded P19: From irrelevance to elegance. *Nature Reviews Microbiology, 4*(5), 405-411.

Selvarajan, R., Mary Sheeba, M., Balasubramanian, V., Rajmohan, R., Dhevi, N. L., & Sasireka, T. (2010). Molecular characterization of geographically different banana bunchy top virus isolates in India. *Indian Journal of Virology, 21*(2), 110-116.

Shekhawat, U. K. S., Ganapathi, T. R., & Hadapad, A. B. (2012). Transgenic banana plants expressing siRNAs targeted against viral replication initiation gene display high-level resistance to Banana bunchy top virus infection. *Journal of General Virology, 93*(8), 1804-1813.

Shepherd, D. N., Martin, D. P., & Thomson, J. A. (2009). Transgenic strategies for developing crops resistant to geminiviruses. *Plant Science, 176*(1), 1-11.

Shimura, H., Masuta, C., Yoshida, N., Sueda, K., & Suzuki, M. (2013). The 2b protein of Asparagus virus 2 functions as an RNA silencing suppressor against systemic silencing to prove functional synteny with related cucumoviruses. *Virology, 442*(2), 180–188.

Shimura, H., & Pantaleo, V. (2011). Viral induction and suppression of RNA silencing in plants. *Biochimica et Biophysica Acta - Gene Regulatory Mechanisms, 1809*(11-12), 601-612.

Sicard, A., Yvon, M., Timchenko, T., Gronenborn, B., Michalakis, Y., Gutierrez, S., & Blanc, S. (2013). Gene copy number is differentially regulated in a multipartite virus. *Nature Communications, 4*, 2248.

Singh, J., Zhang, X., Stewart, L. R., Mitchell, T., & Qu, F. (2014). Role of double-stranded RNA-binding proteins in RNA silencing and antiviral defense *Plant Virus-Host Interaction: Molecular Approaches and Viral Evolution* (pp. 1-16).

Šíp, V., Širlová, L., & Chrpová, J. (2006). Screening for Barley yellow dwarf virus-resistant barley genotypes by assessment of virus content in inoculated seedlings. *Journal of Phytopathology, 154*(6), 336-342.

Smith, I. K., Vierheller, T. L., & Thorne, C. A. (1988). Assay of glutathione reductase in crude tissue homogenates using 5,5'-dithiobis(2-nitrobenzoic acid). *Analytical Biochemistry, 175*(2), 408-413.

Smith, N. A., & Eamens, A. L. (2012) Isolation and detection of small RNAs from plant tissues. *Vol. 894. Methods in Molecular Biology* (pp. 155-172).

Stanley, J. (1995). Analysis of African cassava mosaic virus recombinants suggests strand nicking occurs within the conserved nonanucleotide motif during the initiation of rolling circle DNA replication. *Virology, 206*(1), 707-712.

Stanley, J., Frischmuth, T., & Ellwood, S. (1990). Defective viral DNA ameliorates symptoms of geminivirus infection in transgenic plants. *Proceedings of the*

National Academy of Sciences of the United States of America, 87(16), 6291-6295.

Stanley, J., & Townsend, R. (1986). Infectious mutants of cassava latent virus generated in vivo from intact recombinant DNA clones containing single copies of the genome. *Nucleic Acids Research, 14*(15), 5981-5998.

Tabara, H., Grishok, A., & Mello, C. C. (1998). RNAi in C. elegans: Soaking in the Genome sequence. *Science, 282*(5388), 430-431.

Timchenko, T., de Kouchkovsky, F., Katul, L., David, C., Vetten, H. J., & Gronenborn, B. (1999). A Single Rep Protein Initiates Replication of Multiple Genome Components of Faba Bean Necrotic Yellows Virus, a Single-Stranded DNA Virus of Plants. *Journal of Virology, 73*(12), 10173-10182.

Timchenko, T., Katul, L., Aronson, M., Vega-Arreguín, J. C., Ramirez, B. C., Vetten, H. J., & Gronenborn, B. (2006). Infectivity of nanovirus DNAs: Induction of disease by cloned genome components of Faba bean necrotic yellows virus. *Journal of General Virology, 87*(6), 1735-1743.

Tohidfar, M., & Khosravi, S. (2015). Transgenic crops with an improved resistance to biotic stresses. A review. *Biotechnology, Agronomy and Society and Environment, 19*(1), 62-70.

Trenado, H. P., Orílio, A. F., Márquez-Martín, B., Moriones, E., & Navas-Castillo, J. (2011). Sweepoviruses cause disease in sweet potato and related Ipomoea spp.: Fulfilling Koch's postulates for a divergent group in the genus Begomovirus. *PLoS ONE, 6*(11), e27329.

Tsao, T. T. (2008). *Towards the Development of Transgenic Banana bunchy top virus (BBTV)-Resistant Banana plants: Interference with Replication.* Thesis, Thesis submitted for the degree of Doctor of Philosophy in the Centre of Tropical Crops and Biocommodities, Queensland University of Technology, Australia.

UNCTAD. (2012). Banana market: Exports and Imports http://r0.unctad.org/infocomm/anglais/banana/market.htm.

Unseld, S., Frischmuth, T., & Jeske, H. (2004). Short deletions in nuclear targeting sequences of African cassava mosaic virus coat protein prevent geminivirus twinned particle formation. *Virology, 318*(1), 90-101.

Valli, A., López-Moya, J. J., & García, J. A. (2001). RNA Silencing and its Suppressors in the Plant-virus Interplay *eLS*: John Wiley & Sons, Ltd 10.1002/9780470015902.a0021261.

van der Krol, A. R., Lenting, P. E., Veenstra, J., van der Meer, I. M., Koes, R. E., Gerats, A. G. M., . and Stuitje, A. R. (1988). An anti-sense chalcone synthase gene in transgenic plants inhibits flower pigmentation. *Nature, 333*(6176), 866-869.

Vanderschuren, H., Akbergenov, R., Pooggin, M., Hohn, T., Gruissem, W., & Zhang, P. (2007). Transgenic cassava resistance to <i>African cassava mosaic virus</i> is enhanced by viral DNA-A bidirectional promoter-derived siRNAs. *Plant Molecular Biology, 64*(5), 549-557.

Vanderschuren, H., Alder, A., Zhang, P., & Gruissem, W. (2009). Dose-dependent RNAi-mediated geminivirus resistance in the tropical root crop cassava. *Plant Molecular Biology, 70*(3), 265-272.

Vanitharani, R., Chellappan, P., & Fauquet, C. M. (2003). Short interfering RNA-mediated interference of gene expression and viral DNA accumulation in cultured plant cells. *Proceedings of the National Academy of Sciences of the United States of America, 100*(16), 9632-9636.

Várallyay, E., & Havelda, Z. (2013). Unrelated viral suppressors of RNA silencing mediate the control of ARGONAUTE1 level. *Molecular Plant Pathology, 14*(6), 567-575.

Varsani, A., Navas-Castillo, J., Moriones, E., Hernández-Zepeda, C., Idris, A., Brown, J., . and Martin, D. (2014). Establishment of three new genera in the family Geminiviridae: Becurtovirus, Eragrovirus and Turncurtovirus. *Archives of Virology, 159*(8), 2193-2203.

Voinnet, O. (2005). Induction and suppression of RNA silencing: Insights from viral infections. *Nature Reviews Genetics, 6*(3), 206-220.

Voinnet, O., Vain, P., Angell, S., & Baulcombe, D. C. (1998). Systemic spread of sequence-specific transgene RNA degradation in plants is initiated by localized introduction of ectopic promoterless DNA. *Cell, 95*(2), 177-187.

Vu, T. V., Roy Choudhury, N., & Mukherjee, S. K. (2013). Transgenic tomato plants expressing artificial microRNAs for silencing the pre-coat and coat proteins of a begomovirus, Tomato leaf curl New Delhi virus, show tolerance to virus infection. *Virus Research, 172*(1–2), 35-45.

Wang, F., Zhang, X., Feng, J., Wang, Z., & Wang, P. (2014). Establishment of a quadruplex real-time PCR for screening of genetically modified tomatoes. *European Food Research and Technology, 238*(4), 683-690.

Wang, X.-L., Chiang, T.-Y., Roux, N., Hao, G., & Ge, X.-J. (2007). Genetic diversity of wild banana (Musa balbisiana Colla) in China as revealed by AFLP markers. *Genetic Resources and Crop Evolution, 54*(5), 1125-1132.

Wang, Y., Cheng, X., Wu, X., Wang, A., & Wu, X. (2014). Characterization of complete genome and small RNA profile of pagoda yellow mosaic associated virus, a novel badnavirus in China. *Virus Research, 188*, 103-108.

Wanitchakorn, R., Harding, R. M., & Dale, J. L. (2000). Sequence variability in the coat protein gene of two groups of banana bunchy top isolates. *Archives of Virology, 145*(3), 593-602.

Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M., Rouse, D. T., Liu, Q., . and Waterhouse, P. M. (2001). Construct design for efficient, effective and high-throughput gene silencing in plants. *The Plant Journal, 27*(6), 581-590.

Wieczorek, P., & Obrępalska-Stęplowska, A. (2014). Suppress to Survive—Implication of Plant Viruses in PTGS. *Plant Molecular Biology Reporter*, 1-12.

Woods, G., & Zito, K. (2008). Preparation of Gene Gun Bullets and Biolistic Transfection of Neurons in Slice Culture. (12), e675.

Wu, C. Y., Lai, Y. C., Lin, N. S., Hsu, Y. H., Tsai, H. T., Liao, J. Y., & Hu, C. C. (2008). A simplified method of constructing infectious clones of begomovirus employing limited restriction enzyme digestion of products of rolling circle amplification. *Journal of Virological Methods, 147*(2), 355-359.

Wu, C. Y., Lai, Y. C., Lin, N. S., Hsu, Y. H., Tsai, H. T., Liao, J. Y., & Hu, C. C. (2008). A simplified method of constructing infectious clones of begomovirus employing limited restriction enzyme digestion of products of rolling circle

amplification. [Research Support, Non-U.S. Gov't]. *Journal of Virological Methods, 147*(2), 355-359.

Xia, Z., Peng, J., Li, Y., Chen, L., Li, S., Zhou, T., & Fan, Z. (2014). Characterization of small interfering rnas derived from sugarcane mosaic virus in infected maize plants by deep sequencing. *PLoS ONE, 9*(5), e97013.

Xiao, B., Yang, X., Ye, C. Y., Liu, Y., Yan, C., Wang, Y., . and Fan, L. (2014). A diverse set of miRNAs responsive to begomovirus-associated betasatellite in Nicotiana benthamiana. *BMC Plant Biology, 14*(1), 10.1186/1471-2229-1114-1160.

Xu, D., Mou, G., Wang, K., & Zhou, G. (2014). MicroRNAs responding to southern rice black-streaked dwarf virus infection and their target genes associated with symptom development in rice. *Virus Research, 190*(0), 60-68.

Xue, B., Guo, J., Que, Y., Fu, Z., Wu, L., & Xu, L. (2014). Selection of suitable endogenous reference genes for relative copy number detection in sugarcane. *International Journal of Molecular Sciences, 15*(5), 8846-8862.

Yang, C. F., Chen, K. C., Cheng, Y. H., Raja, J. A. J., Huang, Y. L., Chien, W. C., & Yeh, S. D. (2014). Generation of marker-free transgenic plants concurrently resistant to a DNA geminivirus and a RNA tospovirus. *Scientific Reports, 4*, e5717.

Younis, A., Siddique, M. I., Kim, C. K., & Lim, K. B. (2014). RNA interference (RNAi) induced gene silencing: A promising approach of hi-tech plant breeding. *International Journal of Biological Sciences, 10*(10), 1150-1158.

Zhang, X., Sato, S., Ye, X., Dorrance, A. E., Jack Morris, T., Clemente, T. E., & Qu, F. (2011). Robust RNAi-based resistance to mixed infection of three viruses in soybean plants expressing separate short hairpins from a single transgene. *Phytopathology, 101*(11), 1264-1269.

Zhang, X., Zhao, P., Wu, K., Zhang, Y., Peng, M., & Liu, Z. (2014). Compositional equivalency of RNAi-mediated virus-resistant transgenic soybean and its nontransgenic counterpart. *Journal of Agricultural and Food Chemistry, 62*(19), 4475-4479.

Zhang, Z. Y., Wang, Y. G., Shen, X. J., Li, L., Zhou, S. F., Li, W. C., & Fu, F. L. (2013). RNA interference-mediated resistance to maize dwarf mosaic virus. *Plant Cell, Tissue and Organ Culture, 113*(3), 571-578.

Zhang, Z. Y., Yang, L., Zhou, S. F., Wang, H. G., Li, W. C., & Fu, F. L. (2011). Improvement of resistance to maize dwarf mosaic virus mediated by transgenic RNA interference. *Journal of Biotechnology, 153*(3-4), 181-187.

Zhao, D., & Song, G. q. (2014). High-throughput sequencing as an effective approach in profiling small RNAs derived from a hairpin RNA expression vector in woody plants. *Plant Science, 228*, 39–47.

APPENDICES

Appendix 1: Media and solutions

Media for Agrobacterium-Mediated Transformation of Banana

YMA/YMB Media: Prepare 1 L

Medium Components	**Amount**
Mannitol	10 g
Yeast extract	0.4 g
K_2HPO_4	1 mL
KH_2PO_4	4 mL
NaCl	1 mL
$MgSO_4.7H_2O$	2 mL
pH	6.8
Antibiotic (add post autoclaving)	

Prepare the following stock solutions (10% (w/v)):
K_2HPO_4
KH_2PO_4
$MgSO_4.7H_2O$
NaCl
Note:
YMA is solid medium (add agar -15 g/L)
YMB is liquid medium (no agar)

Note: Antibiotics to be added at the following concentration in medium:

1. YMA (plates for streaking *Agrobacterium*)

Rifampicin (25 mg/L)

Spectinomycin (100 mg/L)

Carbenicillin (250 mg/L)

2. YMB (broth medium for *Agrobacteium*)

Rifampicin (25 mg/L)

Spectinomycin (100 mg/L)

LB media

Medium Components	**Amount**
Tryptone	10 g
Yeast extract	0.4 g
Agar	1 mL
KH_2PO_4	4 mL

Bacterial re-suspension media (BRM): 1 L

Media components	**Concentration in media**	**Volume (mL)**	**Check**
MS Macro	1/10x	10	
MS Micro	1/10x	1	
MS FeEDTA	1/10x	1	
MS Vitamins	1x	10	
Sucrose	68.4 g/L	68.4 g	
Thiamine (10 mg/mL stock)	0.9 mg/L	90 µL	
Cysteine	0.4 g/L	0.4 g	
Glucose	36 g/L	36 g	
pH		5.3	
Filter sterilize and store at $4^{o}C$			
Acetosyringone (100 mM stock)	100 µM	add just before use	

Co-culture media

CCM (A): 600 mL			CCM (B): 400 mL			
Medium components	**Amount**	**Check**	**Medium Components**	**Concentration in medium**	**Volume (mL)**	**Check**
Sucrose	30 g		MS Macro	1/10x	10	
Maltose	30 g		MS FeEDTA	1/10x	1	
Glucose	10 g		MS Micro	1x	1	
MS Vitamins (100x)	10 mL		pH		5.5	
Myo-Inositol	0.1 g		Make 10 X 40 mL aliquots			
Glutamine	0.1 g		Add agar (0.7 g) to each, autoclave, cool and store at 4^{o}C.			
Malt Extract	0.1 g		**Note:**			
Biotin (1 mg/ mL stock)	1000 µL		Melt 40 mL of CCM (B) and add 60 mL of CCM (A).			
PVP	10 g		Add acetosyringone at a final concentration of 200 µM.			
L-Cysteine	0.4 g					
Ascorbate (10 mg/mL stock)	1000 µL					
Proline	0.3 g					
pH	5.3					
Filter sterilize and store at 4^{o}C.						

Acetosyringone (100 mM stock): Dissolve 19.6 mg powder in 1 mL of DMSO. Use 2 uL per mL of media.

Transformation Protocol

Agro Culture

1. Grow *Agrobacterium tumefaciens* (AGL1) in LB media (1-3 mL) + antibiotics for 3 days at 200 rpm and 28^{o}C.
2. Subculture *Agrobacterium tumefaciens* (AGL1) in fresh LB (20 mL) + antibiotics a day before transformation (24 hours growth: 200 rpm and 28^{o}C)
3. Spin culture for 10 minutes at 5000 rpm at room temperature
4. Re-suspend pellet in 10 mL BRM + Acetosyringone (3',5'-Dimethoxy-4'-Hydroxyacetophenone) (Sigma-Aldrich Castle Hill NSW-Australia) (final concentration of 200 µM) and shake for 2-3 h at 70 rpm and 25^{o}C

Note: Concentration of antibiotics: Rifampicin (25 mg/L), Spectinomycin (100 mg/L), Kanamycin (50 mg/L), Carbenicillin (250 mg/L) all supplied by Sigma-Aldrich Castle Hill NSW-Australia.

Plant Cells

1. Use 4-5 days after subculture cells
2. For suspensions- sieve through 500 µm mesh (not larger or smaller)
3. Pipette out culture cells into Falcon tubes
4. Let cells settle, remove the supernatant and then add 10 mL pre-warmed fresh BL (kept at 45^{o}C)
5. Heat shock cells for 5 minutes at 45-48 ^{o}C

Co-culture plate prep

1. Mix co-culture media CCM (A) and (B)
2. Add acetosyringone at a final concentration of 200 µM; pour plates

Transformation

1. Add *Agrobacterium tumefaciens* (AGL1) culture at $OD600_{nm}$ = 0.5-0.7 for AGL 1 (1:5 suspension cells : Agro cells respectively)
2. Add Pluronic F68 (ThermoFisherScientific, Victoria, Australia) at 0.02% final concentration
3. Centrifuge at 2000 rpm for 10 min

4. Rest cells for 30 min at 27 ^{o}C
5. Pipette out cells to Whatman filter paper placed on co-culture medium in Petri dishes
6. Co-culture at 23 ^{o}C in dark for 3 days

Post co-culture care

7. Wash cells in Recovery media + Timentin (400 mg/L) 6 times (vortex to remove extra bacteria)

Selection (for npt II selection marker use kanamycin)

8. After washing, transfer cells (250 µL) aliquots on Whatman filter paper placed on selection medium (selection medium contains kanamycin and 200 mg/L timentin)
9. After a day look at pART-TEST 7 plate under UV microscope to check for transformation success
10. Subculture every 3-4 weeks to prevent *Agrobacterium tumefaciens* (AGL1) from over growing the cultures.
11. After selection, transfer to regeneration medium containing lower concentrations of kanamycin and timentin (200 mg/L).

Banana Agrobacterium Transformation: related stocks, media and additives

STOCK SOLUTIONS:

MS Stock Solution 1 Macronutrients

10 ×concentrations (Use 100 mL per L) Prepared by

	1 L	**Date and Check**
NH_4NO_3 (g)	16.5	
KNO_3 (g)	19.0	
$CaCl_2$ $2H_2O$ (g)	4.4	
$MgSO_4$ $7H_2O$ (g)	3.7	
KH_2PO_4 (g)	1.7	

MS Stock Solution 2 Micronutrients

Prepare in 1 L 100 × concentration (use 10 mL per L)

	1 L	**Date and Check**
$MnSO_4.H_2O$	1690 mg	
$ZnSO_4$ $7H_2O$	860 mg	
H_3BO_4	620 mg	
KI (1 g/10 mL stock)	830 µl	
$NaMoO_4$ $2H_2O$ (1 g/10 mL stock)	250 µl	
$CuSO_4$ $5H_2O$ (mg)	25 mg	
$CoCl_2$ $6H_2O$ (mg)	25 mg	

MS Stock Solution 4 Fe-EDTA

100 × concentration (Use at 10 mL per L)

	500 mL	**Date and Check**
Na_2EDTA $2H_2O$ (mg)	1862	
$FeSO_4$ $7H_20$ (mg)	1390	

Dissolve Na_2EDTA in 80% of final volume of 95°C water. While stirring, add $FeSO_4$. Keep stirring while allowing solution to cool. Make up to final volume and store in light-proof container.

SH Stock Solution 1 Macronutrients

10 × concentrations (Use 100 mL per L) Prepared by

	1 L	**Date and Check**
$NH_4H_2PO_4$ (g)	3.0	
KNO_3 (g)	25.0	
$CaCl_2$ $2H_2O$ (g)	2.0	
$MgSO_4$ $7H_2O$ (g)	4.0	

SH Stock Solution 2 Micronutrients

Prepare in 1 L 100 × concentration (use 10 mL per L) Prepared by

	1 L	Date and Check
$MnSO_4.H_2O$	1000 mg	
$ZnSO_4$ $7H_2O$	100 mg	
H_3BO_4	500 mg	
KI (1 g/10 mL stock)	1 mL	
$NaMoO_4$ $2H_2O$ (1 g/10 mL stock)	100 µL	
$CuSO_4$ $5H_2O$ (mg)	200 mg	
$CoCl_2$ $6H_2O$ (mg)	100 mg	

SH Stock Solution 4 Fe-EDTA

100 × concentration (Use at 10 mL per L) Prepared by

	500 mL	**Date and Check**
Na_2EDTA $2H_2O$ (mg)	1000	
$FeSO_4$ $7H_20$ (mg)	750	

Dissolve Na_2ETDA in 80% of final volume of 95°C water. While stirring add $FeSO_4$. Keep stirring while allowing solution to cool. Make up to final volume and store in light-proof container.

MS Stock Solution 3 Vitamins

Prepare in 100 mL. 1000 × concentration (use 1 mL per L) Prepared by

	100 mL	**Date and Check**
Myo-inositol (mg)	100 000	
Glycine(mg)	2000	
Nicotinic acid (mg)	500	
Pyridoxine HCl (mg)	500	
Thiamine HCl (mg)	100	

Bluggoe Vitamin Stock

Prepare in 1 L. 100 × concentration (use 10 mL per L) Prepared by

	1 L	**Date and Check**
Glycine (mg)	200	
Nicotinic acid (mg)	50	
Pyridoxine HCl (mg)	50	
Thiamine HCl (mg)	40	

Morel and Wetmore Vitamin Stock (Morel and Wetmore 1951)

Prepare in 1 L. 100 × concentration (use 10 mL per L) Prepared by

	1 L	Date and Check
Myo-inositol (mg)	10,000	
Nicotinic acid (mg)	100	
Pyridoxine HCl (mg)	100	
Thiamine HCl (mg)	100	
Ca Pantothenate (mg)	100	
Biotin (1mg/ml)	1 mL	
Folic Acid (mg)	1000	

MEDIA FOR BANANA TRANSFORMATION AND TISSUE CULTURE

BL initial cell selection media

Ingredients	Quantity 1 L	√ when added
MS stock 1 (Macro) 10X	50 mL	
MS Stock 2 (Micro) 100X	10 mL	
MS stock 4 (Fe EDTA) 100X	5 mL	
Bluggoe vit 100X stock	10 mL	
2,4D (10 mg/mL)	0.2 mL	
Ascorbate	10 mg	
Sucrose	20 g	
pH (KOH/HCl) 5.8 (check when done and write the exact pH) Calibrated pH		
Gelzan	2.5 g in each bottle	

Autoclave at 121^0C/15 min and then keep in water bath to cool down to 55^0C before adding the following filter sterilised additives

BL Initial cell selection media-post autoclaving additives

Ingredients	Quality 1 L	√ when added
Timentin (200 mg/mL)	1 mL in each bottle	
Kanamycin (100 mg/mL)	0.5 mL in each 1 L bottle	
	1 mL in each 1 L bottle	
Zeatin (1 mg/mL)	250 µL in each bottle	

Mix the contents well before dispensing in 30 plates /1 L

M3 (Embryogenesis Media)

Ingredients	Quantity 1 L	√ when added
SH stock 1 (Macro) 10X	100 mL	
SH Stock 2 (Micro) 100X	10 mL	
SH stock 4 (Fe EDTA) 100X	10 mL	
MS Vit 1000X stock	1 mL	
Biotin (1 mg/mL)	1 mL	
L-Glutamine	100 mg	
Proline	230 mg	
Malt Extract	100 mg	
Lactose	10 g	
NAA (1 mg/mL)	200 µL	
Kinetin (1 mg/mL)	100 µL	
Sucrose	45 g	
pH (KOH/HCl) 5.8 (check when done and write the exact pH) Calibrated pH		
Agar	7 g in each bottle	

Autoclave at 121^{0}C/15 min and then keep in water bath to cool down to 55^{0}C before adding the following filter sterilised additives M3 (embryogenesis Media)-

Ingredients	Quality 1 L	√ when added
Timentin (200 mg/mL)	1 mL in each bottle	
Kanamycin (100 mg/mL)	1 mL in each 1 L bottle	
STS (0.02 M) (1 mL made fresh by adding 200 µl of 0.1M silver nitrate stock solution into 800 µl of 0.1 M sodium thiosulfate stock solution)	1 mL in each 1 L bottle	
GA3 (1 mg/mL)(only need for psy1, psy2 and Crtl constructs)	0.5 mL in each bottle	
2iP (1 mL/mL)	140 µL in each bottle	
Zeatin (1 mg/mL)	100 µL in each bottle	

Mix the contents well before dispensing on 30 plates /1 Litre

M4 (Embryo Germination)

Ingredients	Quantity 1 L	√ when added
MS Stock 1 (Macro) 10X	100 mL	
MS Stock 2 (Micro) 100X	10 mL	
MS Stock 4 (Fe EDTA) 100X	10 mL	
More and Westmore Vit 1000X stock	1 mL	
BAP (1 mg/mL)	50 uL	
Sucrose	30 g	
pH (KOH/HCl) 5.8 (check when done and write the exact pH) Calibrated pH		
Gelzan	2.5 g in each bottle	

Autoclave at 121^0C/15 min and then keep in water bath to cool down to 55^0C before adding the following filter sterilised additives

M4 (Embryo Germination) - Post autolaving additives

Ingredients	Quality 1 L	√ when added
Timentin (200 mg/mL)	1 mL in each bottle	
Kanamycin (100 mg/mL)	1 mL in each 1 L bottle	
STS(0.02M) (1 mL made fresh by adding 200 µl of 0.1 M silver nitrate stock solution into 800 µL of 0.1 M sodium thiosulfate stock solution)	1 mL in each 1 L bottle	
IAA (1 mg/mL)	200 µL in each bottle	

Mix the contents well before dispensing on 30 plates /1 L

M5 (Rooting Media)

Ingredients	Quantity 1 L	√ when added
MSstock 1 (Macro) 10X	100 mL	
MS Stock 2 (Micro) 100X	10 mL	
MS stock 4 (Fe EDTA) 100X	10 mL	
More and westmore Vit 1000X stock	1 mL	
Sucrose	30 g	
pH (KOH/HCl) 5.8 (check when done and write the exact pH) Calibrated pH		
Gelzan	2 g in each bottle	

Autoclave at 121^0C/15 min and then keep in water bath to cool down to 55^0C before adding the following filter sterilised additives

M5 (Rooting)- Post autoclaving additives

Ingredients	Quality 1 L	√ when added
Timentin (200 mg/mL)	1 mL in each bottle	
Kanamycin (100 mg/mL)	1 mL in each 1 L bottle	
	2 mL in each bottle (for K200 media)	
STS(0.02 M) (1ml made fresh by adding 200 µL of 0.1 M silver nitrate stock solution into 800 µL of 0.1 M sodium thiosulfate stock solution)	1 mL in each 1 L bottle	

Mix the contents well before dispensing on 20 pots or 30 tubes /1 L

Multiplication media

Ingredients	Quantity 1 L	√ when added
MSstock 1 (Macro) 10X	100 mL	
MS Stock 2 (Micro) 100X	10 mL	
MS stock 4 (Fe EDTA) 100X	10 mL	
MS Vit 1000X stock	1 mL	
BAP (1mg/mL)	2.5 mL	
Sucrose	20 g	
pH (KOH/HCl) 5.8 (check when done and write the exact pH) Calibrated pH		
Gelzan	2 g in each bottle	

Autoclave at 121^0C/15 min and then keep in water bath to cool down to 55^0C before adding the following filter sterilised additives

Mix the contents well before dispensing on 20 pots /1 L

Note: if using the Multiplication media from Phytotech M491 add BAP, Sucrose and Gelzan or agar.

Appendix 2: PCR and Southern blot analyses

Additional figures for chapter 3

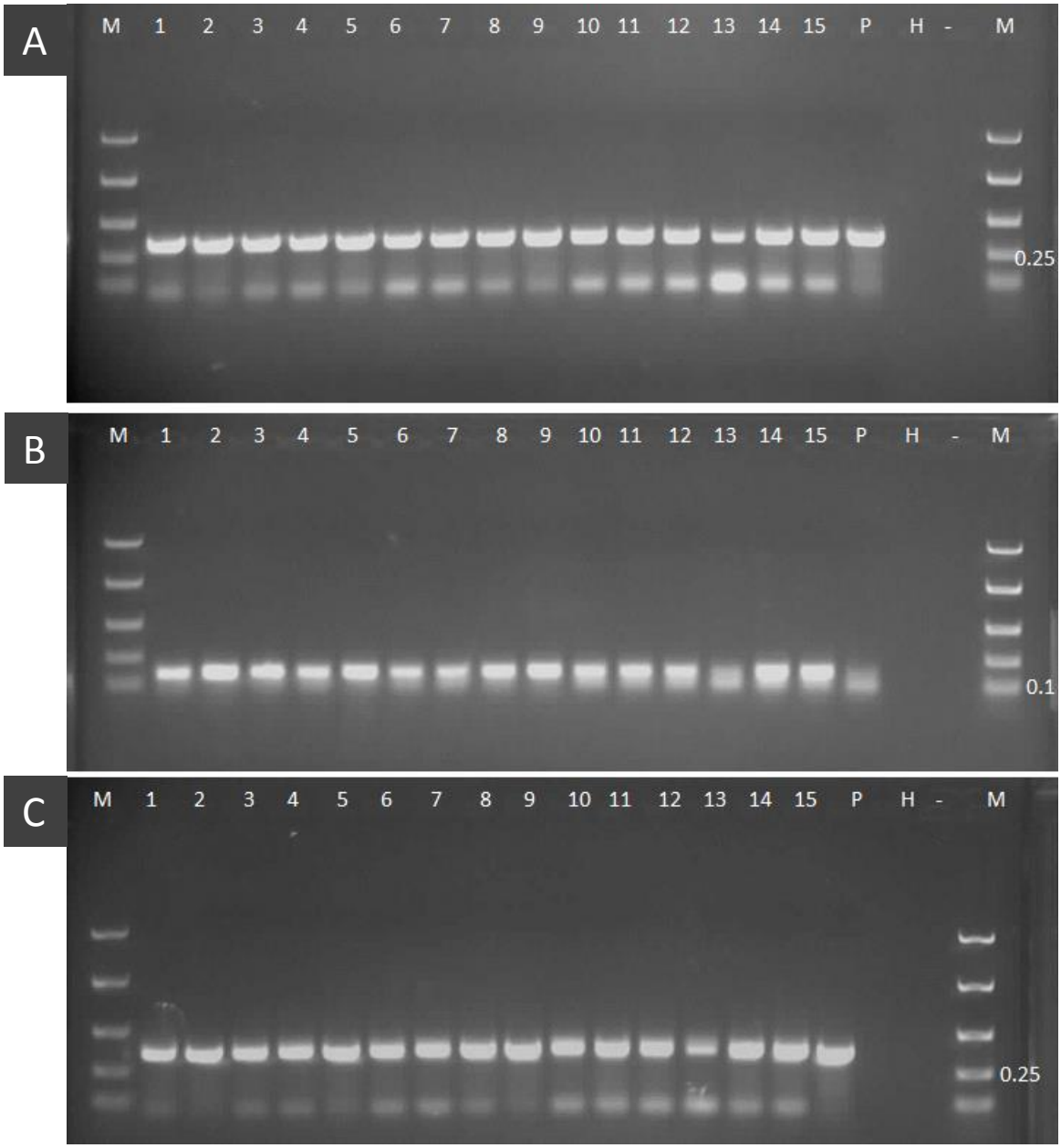


Figure 1: PCR analysis of SP-MP putative transgenic lines from transformation experiment 2. A) Sense arm PCR products from lines SP-MP1-15; and B) syntron PCR products and C) Antisense arm PCR products from lines SP-MP1-15. '-' is no template control, 'H' is wild type control and '+' is the plasmid DNA positive control. Expected size of the PCR product is 340 bp.

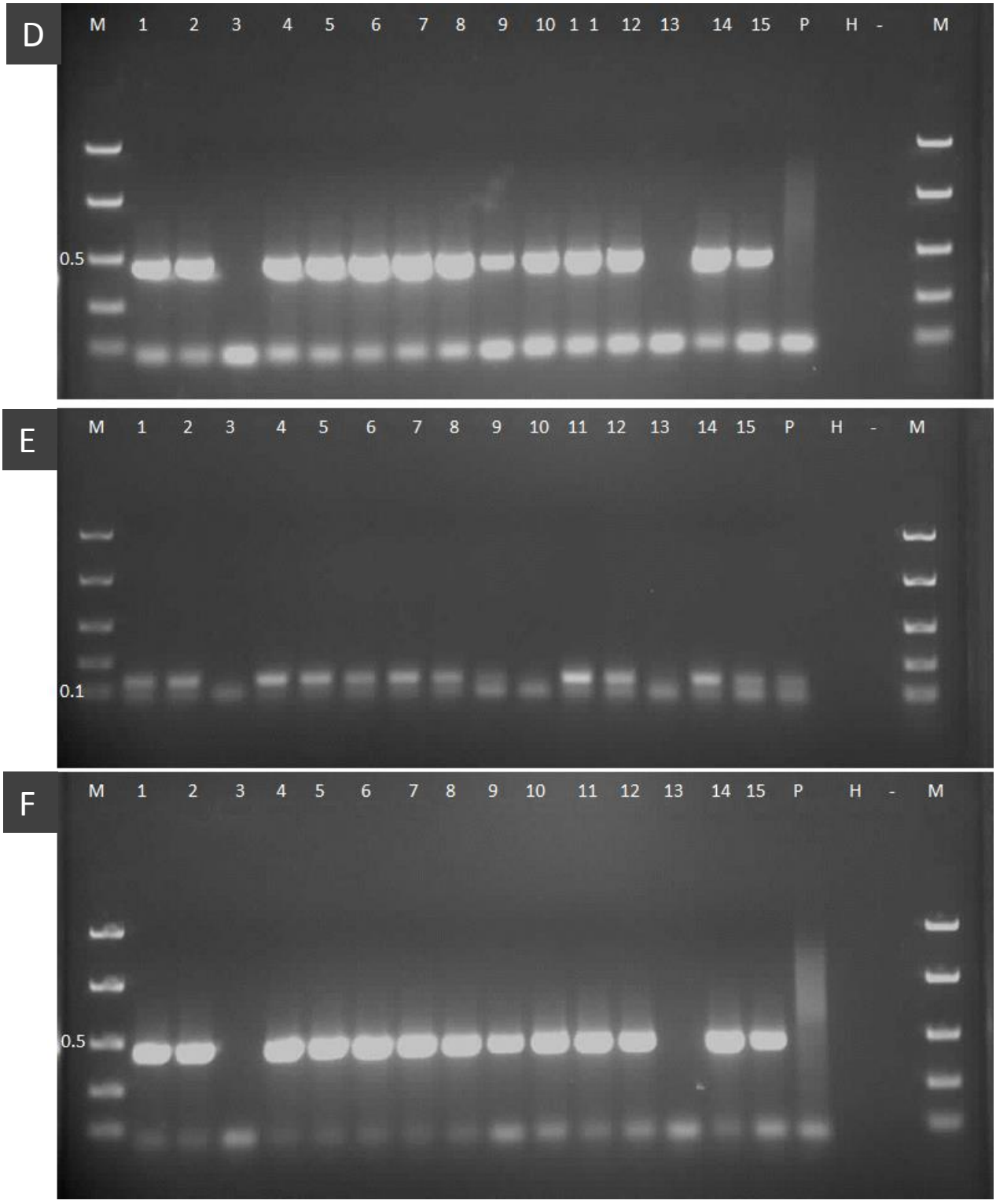


Figure 2: PCR analysis of representative SP-Rep5'NSP putative transgenic lines from transformation experiment 2. D) Sense arm PCR products from lines SP- Rep5'NSP1-15; and E) Syntron PCR products and F) Antisense arm PCR products from lines SP- Rep5'NSP1-15. '-' is no template control, 'H' is wild type control and '+' is the plasmid DNA positive control. Expected size of the PCR product is 431 bp.

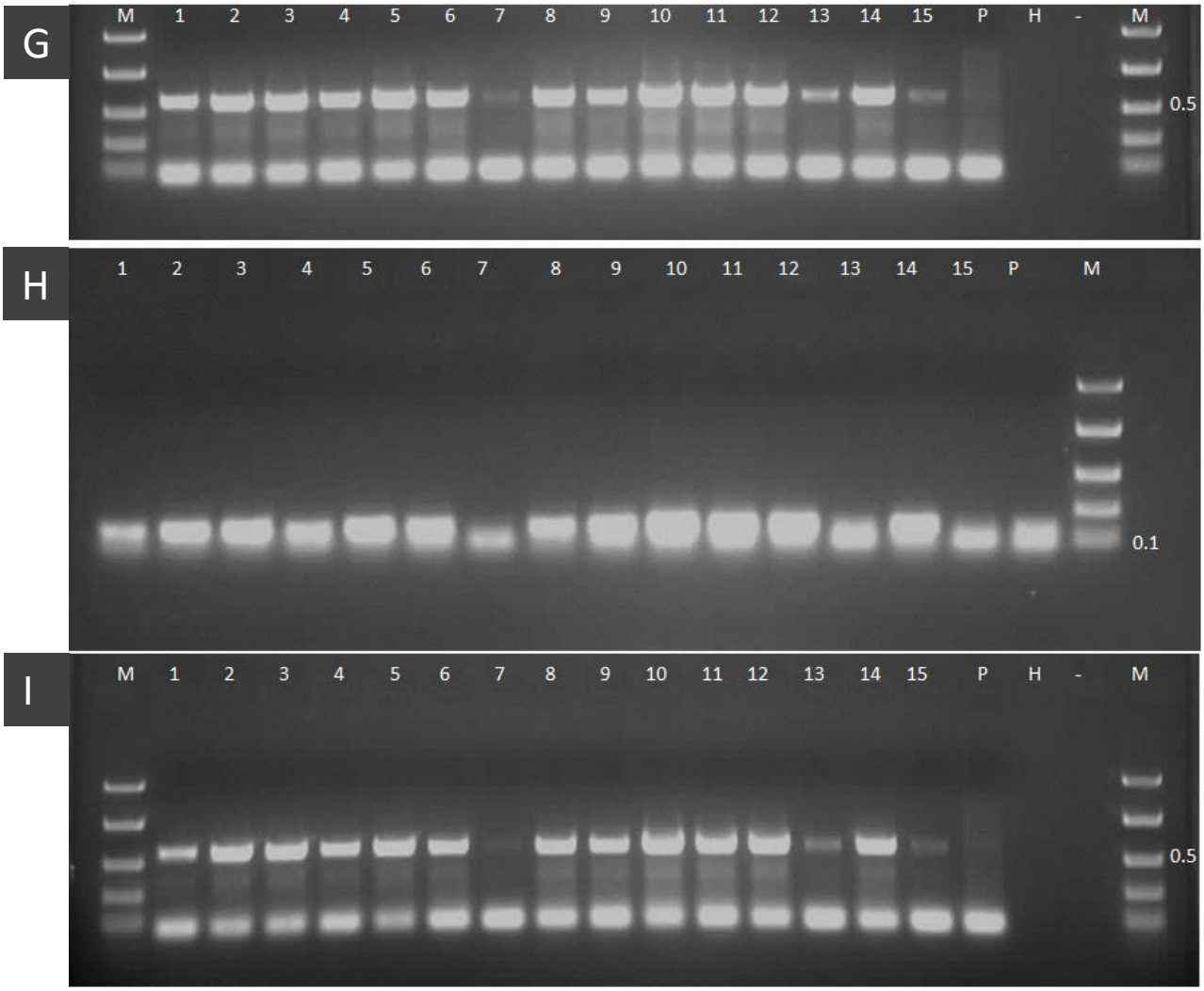


Figure 3: PCR analysis of representative SP-Rep5'MP putative transgenic lines from transformation experiment 2. G) Sense arm PCR products from lines SP- Rep5'MP1-15; and H) Syntron PCR products and I) Antisense arm PCR products from lines SP- Rep5'MP1-15. '-' is no template control, 'H' is wild type control and '+' is the plasmid DNA positive control. Expected size of the PCR product is 620 bp. M is the EasyLadder (Bioline).

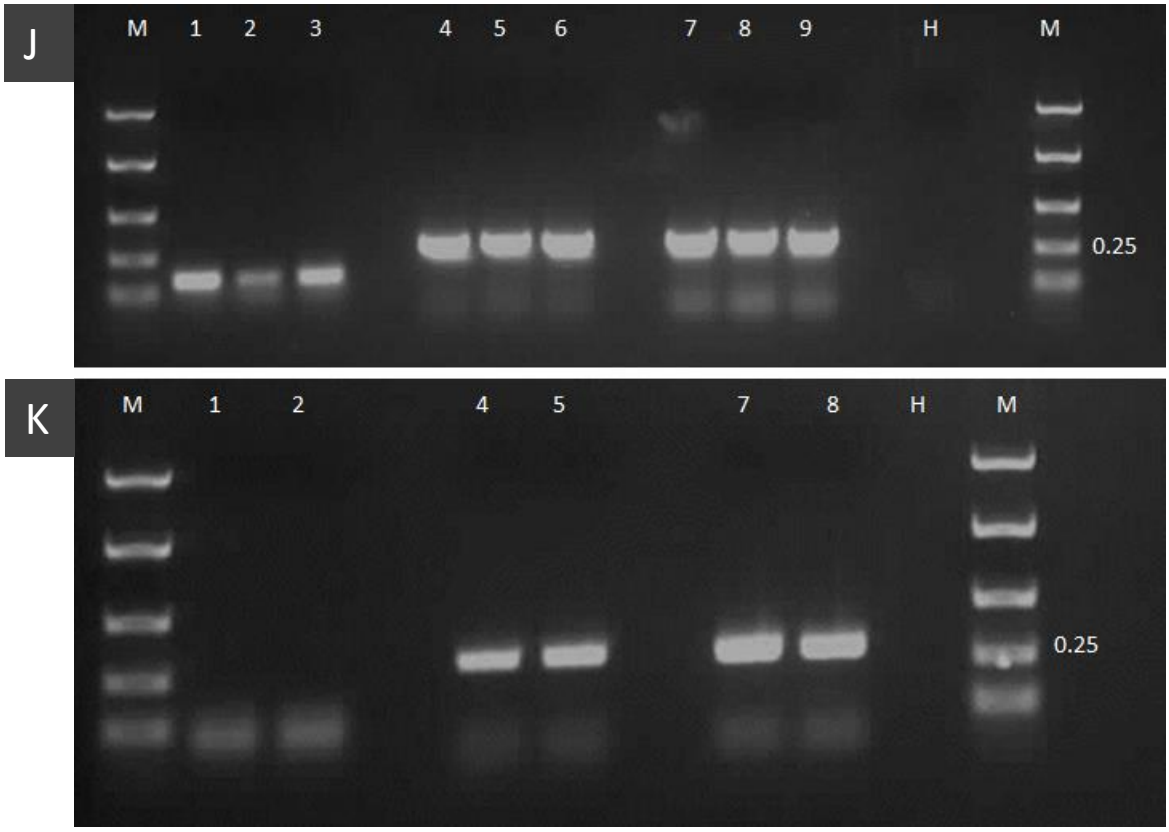


Figure 4: PCR analysis of representative SP-Rep5' (J) and SP-RepIO (K) putative transgenic lines from transformation experiment 2. J) Sintron PCR products (1-3) Sense arm PCR products from lines SP-Rep5'(4-6); and Antisense arm PCR products from lines SP- Rep5'(7-9). K) Sintron PCR products (1-2), Sense arm PCR products from lines SP-RepIO'(3-4); and Antisense arm PCR products from lines SP- RepIO(5-6). 'H' is wild type control. Expected size of the PCR product is 280 (J) and 279 (K) bp. M is the EasyLadder (Bioline).

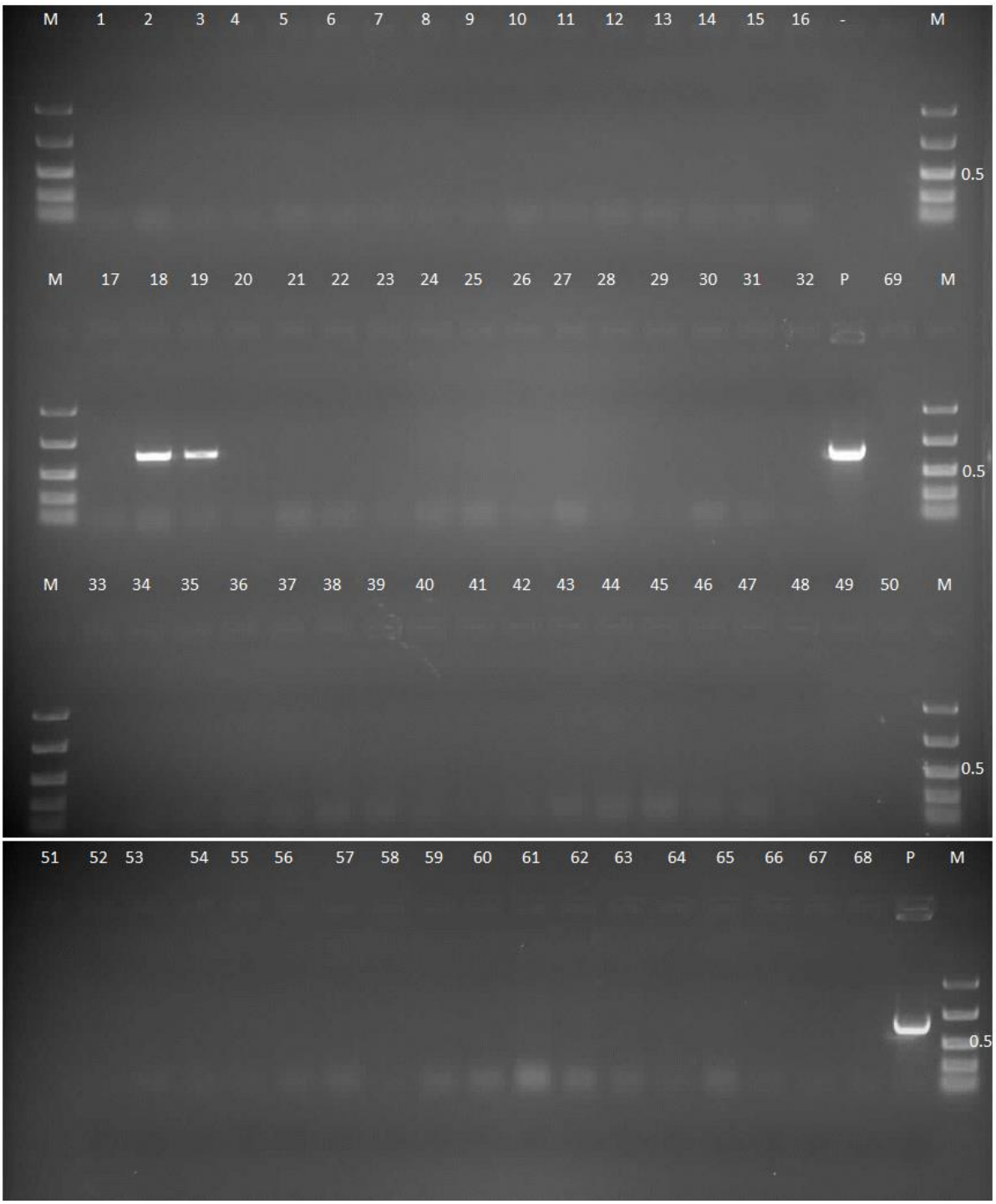


Figure 5: PCR analysis of SP-MP (1-16), SP-Rep5'MP (17-32), SP-NSP (33-47), SP-Rep5' (48-50), SP-Rep5'NSP (51-65), SP-RepIO (66-67), SP-RepIONSP (68) and SP-RepIOMP (69) putative transgenic lines from transformation experiment 2 for the presence of VirC gene from Agrobacterium'-' is non template control. Expected size of the PCR product is 600 bp. M is the EasyLadder (Bioline). Vir C gene presence was detected in 2 plants of SP-Rep5'MP which were replaced with new lines which were also screened and confirmed to be free of Agrobacterium contamination.

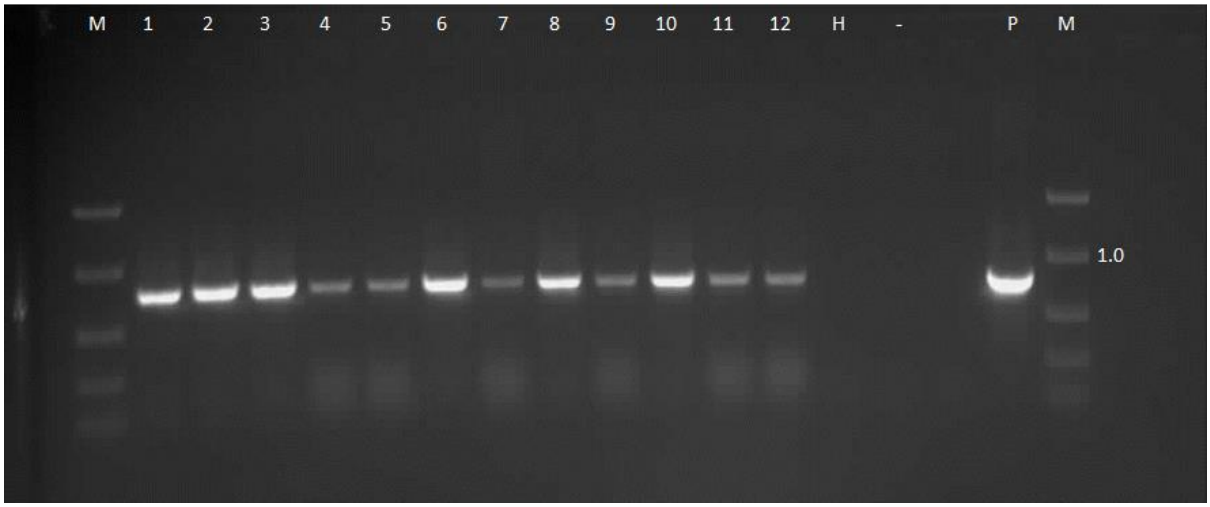


Figure 6: RT-PCR analysis of SP-Reo5'MP transgenic banana lines from transformation experiment 2. The primers used amplify a 722 bp region of the hairpin arm and syntron. Lane '-' no template control, 'P' positive control-plasmid DNA harbouring the SP-MP construct, 'H' wild-type control and 'M' is EasyLadder 1 (Bioline).

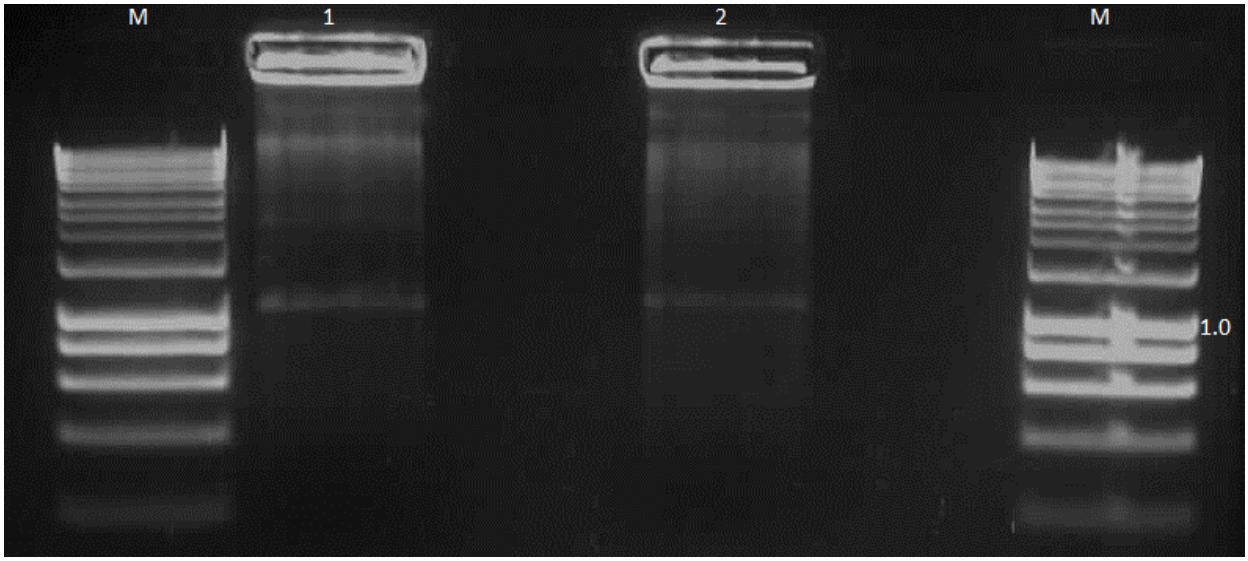


Figure 7: Agarose gel electrophoresis of BBTV DNA-C and –N digested using *MfeI* and *PacI* respectively. Lanes 1-2 represent BBTV DNA-C and -N. Digest products of ~1.1 kb are present representing linearised, full-length monomeric DNA of each component. M is EasyLadder 1 (Bioline).

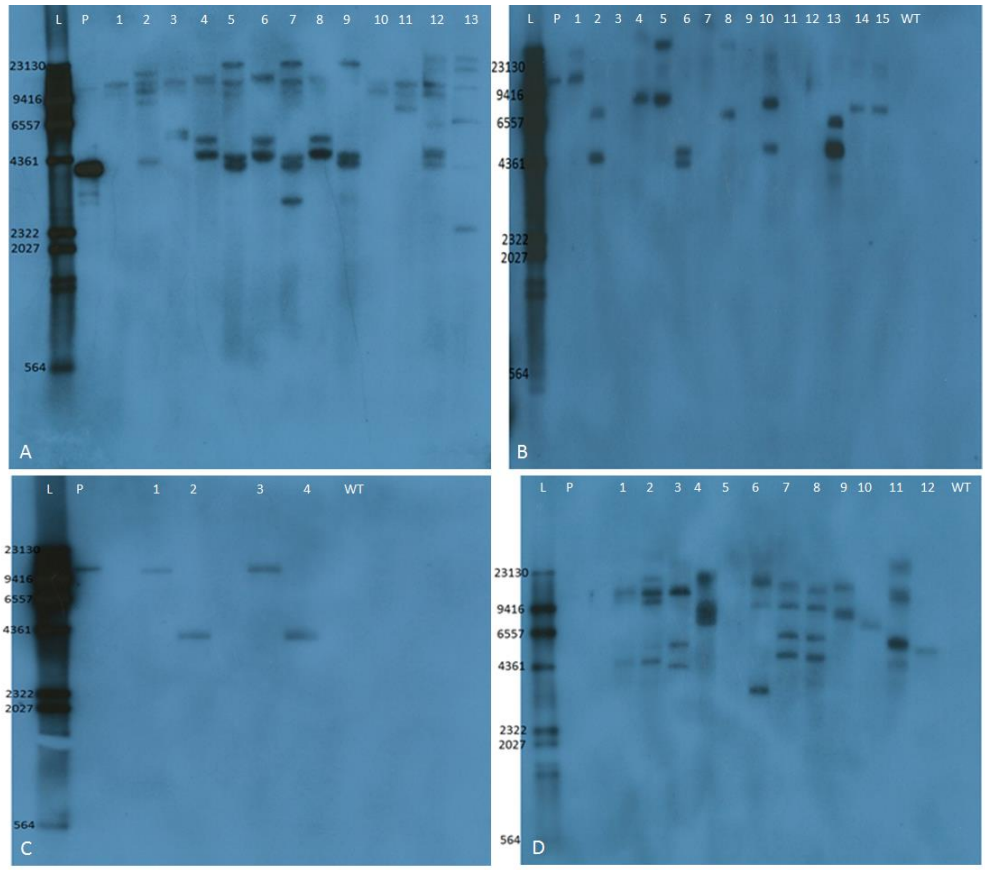


Figure 8: Representative results of Southern blotting of transgenic banana lines transformed with NSP, SP-Rep5'NSP, SP-MP and SP-Rep5'MP RNAi constructs using the DIG labelled NPTII probe. 'M' - is the DIG-labelled molecular marker, '+' is the positive control (50 ng of plasmid DNA), 'WT' is non-transgenic control; A) lanes 1-11 represent SP-NSP transgenic lines (note – not all lines from this construct are shown in the figure); B) lanes labelled 1-15 represent SP-Rep5'NSP transgenic lines. C) SP-MP-2 lines digested with BamHI(1/3) and HindII (2/4) showing single copy transgene integration. Independent transgenic lines are characterised by differing hybridisation signal patterns and/or a varying number of transgene copies. In Figure D (SP-Rep5'MPlines) lanes 8 and 10 are not independent as they have the same hybridisation signal patterns.